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Bacterial Protein Toxins

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Preface

In recent years, remarkable progress has been accomplished with respect to our knowledge about bacterial protein toxins. This refers especially to structural aspects of protein toxins but holds also true for genetics, molecular biology and biochemical mechanisms underlying the action of toxins. The great advances made in the field of bacterial toxinology have depended on fruitful interdisciplinary efforts, which have brought together approaches from microbiology, cell biology and structural biochemistry, and not least from pharmacology. Beyond question, a significant contribution to the understanding of toxins comes from crystal structure analyses. Thus, the crystal structures of many of the classical toxins have been solved during recent years, including those for diphtheria toxin, cholera toxin, pertussis toxin, botulinum neurotoxins and many pore-forming toxins. Methodological interactions of microbiologists and cell biologists have been extremely successful and have resulted in the establishment of a new discipline termed "cellular microbiology", defining the events and consequences of the interaction of bacteria and/or toxins (or other bacterial products) with host or target cells.

Pharmacologists have been protagonists of "toxinology" for a long time and, in particular, molecular pharmacologists have contributed significantly to the advances in the field of bacterial protein toxins. The renaissance in interest of pharmacologists in toxins results from the fact that, today, experimental pharmacology has been focused on "signalling" in its broadest sense. Rather early, bacterial protein toxins have been recognised as important pharmacological tools in signal transduction research. The reason for this fact is evident. First, the bacterial protein toxins are often extremely potent agents. Second, they act with remarkably high selectivity and specificity. Finally, studies from recent years showed that targets of bacterial toxins are quite often essential elements of cell signal transduction pathways. Broad usage of toxins as tools was initiated by the elucidation of the actions of cholera and pertussis toxins on heterotrimeric G proteins. This type of application of toxins was renewed recently by the findings that various bacterial toxins, including C3-like exoenzymes, the large clostridial cytotoxins and the cytotoxic necrotizing factors (CNFs) act selectively on small GTPases of the Rho/Ras family and potently inactivate and activate, respectively, these eukaryotic signal switches. Thus, C3

exoenzyme from *Clostridium botulinum* played an essential role in deciphering the function of Rho GTPases in regulation of the actin cytoskeleton through membrane receptors.

However, bacterial toxins are not only important in experimental pharmacology but also in pharmacotherapy for humans. For example, the knowledge of the exact mode of action of the botulinum neurotoxins approved them as drugs for so-far difficult-to-treat diseases. Moreover, fusion protein toxins using endogenous ligands for tissue-specific targeting of cytocidal toxins are an intriguing approach for treating, for example, special entities of cancer or autoimmune diseases.

This volume covers, we are convinced, the very current and exciting aspects of up-to-date bacterial toxinology and comprehensively reviews the most important bacterial protein toxins. It begins with a review on the various cell entry mechanisms of protein toxins followed by several chapters on ADP-ribosylating toxins, which are still the best-understood bacterial toxins. These chapters summarise recent progress in the structure–function analysis of ADP-ribosylating toxins and comprehensively review the "classical" ADP-ribosylating toxins including diphtheria toxin, *Pseudomonas* exotoxin A, cholera toxin and pertussis toxin, which modify elongation factor 2 and heterotrimeric G proteins, respectively. In addition, also bacterial toxins that ADP-ribosylate small GTPases (C3 exoenzymes and *Pseudomonas* exoenzyme S) and actin (e.g. *C. botulinum* C2 toxin) are covered. Complementing chapters are devoted to ADP-ribosylating factors (ARFs), practical application of pertussis toxin as a pharmacological tool and diphtheria toxin-based fusion toxins. Further topics of the volume are the recently discovered new family of large clostridial cytotoxins, which modify small GTPases of the Rho/Ras family by glucosylation and the cytotoxic necrotizing factor (CNF) from *Escherichia coli* that causes constitutive activation of Rho GTPases by deamidation. The bacterial toxins that act as metalloproteases (e.g. clostridial neurotoxins and anthrax toxin) are extensively reviewed with respect to recent findings on structure and function relationship, cell entry mechanisms and modes of action. Pore-forming toxins and potentially haemolytic toxins are the topic of a series of articles that review the typical pore-forming or membrane-damaging toxins, such as *Staphylococcal* alpha-toxin and bacterial phospholipases, and describe in detail their usage as pharmacological tools. Additional toxins reviewed are the invasive adenylate cyclase toxin from *Bordetella pertussis,* the vacuolating cytotoxin from *Helicobacter pylori*, the *Escherichia coli* heat-stable enterotoxins and Shiga toxins. A rather new family of extracellularly acting toxins, the group of superantigens, are reviewed and discussed with respect to structure, functions and pathogenetic roles. A comprehensive review on the structure and activity of endotoxins is included and, last not least, recent exciting findings on bacterial toxic factors produced by *Yersinia*, which appear to emerge to a new category of protein toxins, are discussed in the last chapter of the volume.

The aim of this volume of the Handbook of Experimental Pharmacology is to give a review and an update of the current knowledge on bacterial protein toxins presented by different disciplines and is intended to be used by scientists from diverse scientific fields.

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CHAPTER 1

Uptake of Protein Toxins Acting Inside Cells

S. OLSNES, J. WESCHE, and P.Ø. FALNES

A. Introduction and Brief Description of Relevant Toxins

Bacterial protein toxins act either at the level of the cell surface or on targets in the cytosol. Superantigens and toxins containing lytic activity exert their action at the level of the cell surface and will not be discussed here. However, an increasing number of bacterial protein toxins are being demonstrated to have intracellular sites of action. This implies that the toxins must be able to cross cellular membranes. Most toxins do this by crossing membranes of intracellular organelles rather than penetrating the plasma membrane.

Some toxins are equipped with their own translocation apparatus, whereas others must rely on cellular translocation processes that they exploit. This is most clearly evident in the case of immunotoxins. These consist of an antibody that binds to target cells and a protein that is highly toxic once in the cytosol but that is unable to translocate to the cytosol on its own. Recent research has shed some light on how these toxins and immunotoxins enter the cytosol.

The toxins that will be particularly considered here are depicted schematically in Fig. 1. Diphtheria toxin is synthesized as a single-chain protein with a signal sequence that is cleaved off when the toxin is excreted from the bacteria. The mature toxin is easily cleaved into two fragments by trypsin-like proteases, and the cleaved toxin represents the active form. It consists of an enzymatically active part, the A fragment, which is linked by a disulfide bond to the B fragment (PAPPENHEIMER 1977). The B fragment consists of a receptor-binding domain (R) and a transmembrane domain (T), which can insert into the membrane and plays a decisive role in the translocation of the A fragment to the cytosol (CHOE et al. 1992). It resembles membrane-inserting domains in certain colicins and in the δ-toxin from *Bacillus thuringensis* (LI et al. 1991). Once in the cytosol, the A fragment adenine diphosphate (ADP) ribosylates a unique amino acid, diphthamide, in elongation factor 2, thereby inactivating the elongation factor and inhibiting protein synthesis (COLLIER 1975; VAN NESS et al. 1980a, 1980b).

In many respects, *Pseudomonas aeruginosa* exotoxin A resembles diphtheria toxin. It has an enzymatically active part (domain III) that has the same activity as diphtheria-toxin A fragment and a region (domain II) that is structurally reminiscent the T-domain of diphtheria toxin and which is, therefore, believed to be involved in the translocation process (WICK et al. 1990). Unlike diphtheria

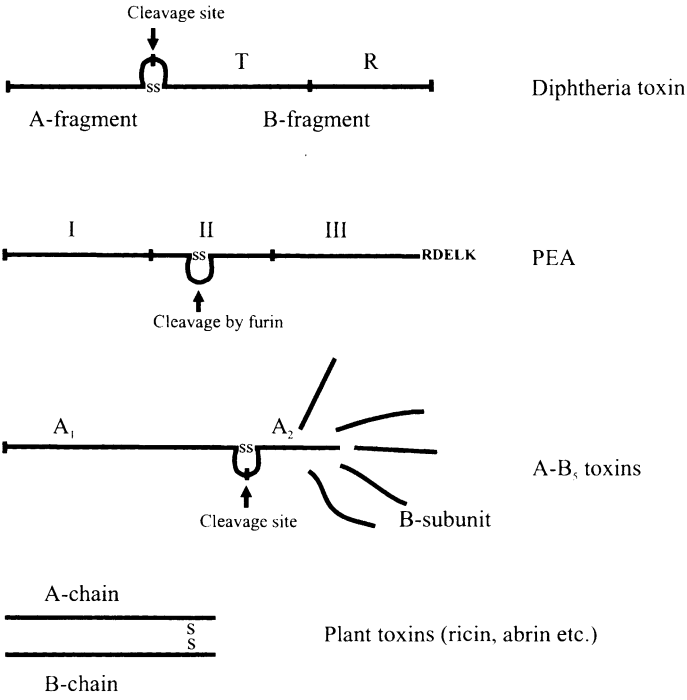

Fig. 1. Schematic structure of toxins. Details are discussed in the text

toxin and most other toxins in this group, the enzymatically active domain of exotoxin A is located in the C-terminal part of the molecule. A disulfide-bridged cleavage site sensitive to furin is located in domain II, and it is believed that cleavage and reduction is required for translocation (ALLURED et al. 1986). Domain I is responsible for binding the toxin to cell-surface receptors.

The A–B_5 class of toxins comprises cholera toxin, *Escherichia coli* heat labile toxin, Shigella toxin and Shiga-like toxins 1 and 2 (MERRITT and HOL 1995). These toxins have a similar overall structure, but they have different functions. The toxins all consist of a pentameric B subunit that is linked non-covalently to the C-terminal part of the A subunit, which consists of a single polypeptide chain. In its C-terminal end, the A subunit contains a disulfide-bridged trypsin- and furin-sensitive loop, which is easily cleaved to yield the enzymatically active A_1 fragment and the small A_2 fragment, which is associated with the B-subunit (OLSNES et al. 1981; REISBIG et al. 1981). The B subunit of all toxins in this group binds to glycolipids. The enzymatically active A subunits of cholera toxin and heat-labile toxin ADP ribosylate and permanently activate the α-subunit of trimeric G-proteins, whereas the A-subunit of Shigella toxin and Shiga-like toxins inactivate ribosomes by removal of an adenine residue from the 28S RNA of the large ribosomal subunit, thereby inhibiting protein synthesis.

We will also discuss a group of plant toxins comprising ricin, abrin, modeccin, volkensin and others that have properties similar to the bacterial toxins we are considering here (Olsnes et al. 1974). These toxins are synthesized as a continuous polypeptide chain but, prior to excretion, they are cleaved in the vacuole of the plant cells to yield an enzymatically active A chain and a receptor-binding B chain held together by a disulfide bond (Fig. 1). The B chain has lectin-like properties and binds to surface glycoproteins and glycolipids with terminal galactose residues (Olsnes and Refsnes 1978; Sandvig and Olsnes 1979). The A chain has the same enzymatic activity as the A subunit of Shiga toxin (Endo et al. 1988).

The binary toxins are produced as two different proteins, one binds to receptors at the cell's surface and is then cleaved to release an approximately 20-kDa fragment. In the case of anthrax toxin (and probably other binary toxins), the remaining major part of the cleaved molecules forms heptamers that act as binding sites for an enzymatically active protein that is able to enter the cytosol. The *Clostridium botulinum* C2 toxin, *C. perfringens iota* toxin and *C. spiriforme* toxin are binary toxins that act by ADP ribosylating the adenosine triphosphate (ATP)-binding protein actin. There are at least six mammalian actin isoforms, but *C. botulinum* C2 toxin ADP ribosylates only the cytoplasmic form (Aktories et al. 1986, 1992). Anthrax toxin is the most studied binary toxin. In this case, two different enzymatically active proteins, the edema factor and the lethal factor, can use the same binding subunit (called PA or protective antigen) to which they bind in a mutually exclusive manner. The lethal factor is a metalloprotease that cleaves mitogen-activated protein kinase kinase (Duesbery et al. 1998; Vitale et al. 1998). The edema factor is an adenylate cyclase that enters the cytosol where it is activated by calmodulin (Leppla 1982), leading to elevation of the level of cyclic adenosine monophosphate (cAMP), thus interfering with the bacteriocidal capacity of the intoxicated cell.

Another invasive and calmodulin-activated adenylate cyclase (CyaA) is produced by *Bordetella pertussis* (Rogel et al. 1991). The 177-kDa CyaA protein can be divided into an N-terminal catalytic domain and a C-terminal hemolytic domain. Upon activation by calmodulin, CyaA elevates the level of cAMP in the cytosol.

An export protein from *C. botulinum*, exoenzyme C3, is able to ADP ribosylate the small G protein Rho, which regulates actin polymerization and intracellular vesicular transport (Chardin et al. 1989). Similar enzymes are found in a wide variety of bacteria (Just et al. 1992a, 1992b), including *Staphylococcus aureus* (Inoue et al. 1991), although no bacterial substrate is known. Use of exoenzyme C3 has played an important role in the recent elucidation of the mechanism of action of certain microbial toxins, the *C. difficile* toxins A and B (Just et al. 1995a) and the *E. coli* necrotizing toxin [cytotoxic necrotizing factor (CNF1) Lemichez et al. 1997]. When Rho had been pretreated with *C. difficile* toxins, it could no longer be ADP ribosylated by exoenzyme C3, clearly indicating that the *C. difficile* toxin had modified Rho. This modification

was later found to consist of transfer of glucose from the precursor uridine diphosphate–Glc to a threonine residue in Rho and the related Rac and Cdc 42 (Just et al. 1995a, 1995b). Lethal toxin from *C. sordellii* has the same activity, which leads to the disassembly of stress fibers in the toxin-treated cells (Popoff et al. 1996; Selzer et al. 1996). The toxins did not modify the more distantly related G-proteins, rab and ARF. *C. novyi* toxin incorporates *N*-acetylglucosamine rather than glucose into rho (Just et al. 1996).

CNF1 from certain strains of *E. coli* is another toxin acting on Rho. The toxin consists of a single polypeptide chain (137kDa) with a putative N-terminal receptor-binding domain, a central hydrophobic domain and a C-terminal catalytic domain (Lemichez et al. 1997). The toxin induces deamidation of a glutamine residue (Gln63; Flatau et al. 1997; Schmidt et al. 1997), leading to permanent activation of Rho. As a result, there is a strong overactivity in actin polymerization, and formation of multinucleated cells. Furthermore, the toxin induces phagocytosis in non-phagocytic cells, enabling toxigenic bacteria to enter and multiply in cells and to be transcytosed across epithelia. It is apparently due to part of the same strategy that the toxin inhibits apoptosis in the intoxicated cells (Fiorentini et al. 1997).

The neurotoxins tetanus toxin and botulinum toxins are not produced as export proteins but rather as intracellular proteins that are released when the bacterium dies. The molecular action of these toxins has been elucidated in great detail in recent years. They are all metalloproteases that cleave proteins involved in exocytosis, particularly in the synapses (Schiavo et al. 1992; Montecucco and Schiavo 1993). As a result, synaptic transmission is paralyzed. In addition, these toxins are formed as single polypeptide chains that are cleaved into an enzymatically active fragment and a fragment that is involved in binding and translocation. As in the case of diphtheria toxin, the two parts are disulfide linked. Binding to different surface receptors is probably the reason that different neurons are affected in tetanus and botulism.

B. Binding to Cell-Surface Receptors

All bacterial protein toxins must bind to cells in order to act. Since, for obvious reasons, the cells have not developed receptors for toxins, the toxins must employ cell-surface structures that are designed for other purposes. Cells lacking the relevant receptors are resistant to the toxin. The surface structures that the toxins use for binding may be hormone receptors, growth-factor precursors, glycolipids and other molecules. So far, no particular property that is common for toxin receptors has emerged. However, it is likely that the ability to transport the toxin to defined intracellular compartments may prove to be a common property.

Diphtheria toxin binds to the uncleaved precursor of a growth factor, namely that of heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF; Naglich et al. 1992). All growth factors of the EGF family

are formed as transmembrane proteins which are transported out of the cells and then cleaved to release the growth factor. Not all molecules are cleaved, and it is now clear that, in many cases, the uncleaved molecules act as growth factors in a juxtacrine manner by binding to EGF receptors on adjacent cells (RAAB and KLAGSBRUN 1997; TAKEMURA et al. 1997). Diphtheria toxin binds to the HB-EGF precursor present on most mammalian cells. Due to a few amino acid differences, the HB-EGF precursor found in mouse and rat cells does not bind the toxin, and cells from these animals are, therefore, resistant to diphtheria toxin (HOOPER and EIDELS 1996).

Pseudomonas exotoxin A binds to a cell-surface protein that binds low-density lipoprotein and α_2-macroglobulin (KOUNNAS et al. 1992). Cholera toxin and *E. coli* heat-labile toxin bind to the ganglioside Gm_1 (VAN HEYNINGEN 1974), whereas Shigella (or Shiga) toxin and the related Shiga-like toxins bind to neutral glycolipids. Shigella toxin, the essentially identical Shiga-like toxin 1 and the slightly different Shiga-like toxin 2 bind to globoside B_3 (LING et al. 1998), whereas the related Shiga-like 2e toxin binds to globoside B_4 (DEGRANDIS et al. 1989).

One of the botulinum toxins, toxin B, binds to the neuronal protein synaptotagmin (NISHIKI et al. 1994). The receptors for the other botulinum toxins have not been identified.

The plant toxins ricin, abrin and related toxins are rather unselective, as they bind both to glycoproteins and glycolipids with terminal galactose residues (HUGHES and GARDAS 1976; MEAGER et al. 1976; OLSNES and REFSNES 1978). Therefore, it is not clear which molecules are the productive receptors, as it is likely that binding to most of the structures carrying terminal galactose will not result in translocation of the A chain to the cytosol. In the case of anthrax toxin, tetanus toxin and most other protein toxins that enter the cytosol, the nature of the receptors is not known.

C. Endocytosis

Since most toxins that act on components in the cytosol are translocated from intracellular organelles, the uptake mechanism usually starts with endocytosis. The uptake may follow the classical route from coated pits but, in several cases, it appears to occur from other surface structures. These may include caveoli or other, so far uncharacterized, invaginations of the plasma membrane.

The first indication that endocytosis may be necessary for entry of toxins came from experiments in reticulocytes with the plant toxins ricin and abrin (OLSNES et al. 1974). The ribosomes of reticulocytes are very sensitive to abrin and ricin but, in spite of this, protein synthesis in whole reticulocytes was not inhibited after incubation with high concentrations of these toxins. Both toxins were found to bind extensively to the surface of the reticulocytes, but there was no evidence for endocytic uptake. More definitive evidence was obtained with diphtheria toxin in nucleated cells in culture. It was found that, when the cells were incubated with ammonium chloride or other weak bases that are

able to penetrate cellular membranes and neutralize acidic compartments in the cells, the cells were protected from intoxication (Kim and Groman 1965). This protection was overcome when the cells were briefly exposed to low pH (Draper and Simon 1980; Sandvig and Olsnes 1980). Moreover, in the latter case, the cells were more rapidly intoxicated than when the cells were incubated with toxin under normal conditions. The interpretation of these results was that the normal entry pathway for the toxin is from intracellular acidic compartments and that, when cells with surface-bound toxin are exposed to low pH, the conditions in endosomes are mimicked at the level of the surface membrane, and direct translocation is induced.

Later, similar data was obtained with anthrax toxin (Koehler and Collier 1991; Milne and Collier 1993; Wesche et al. 1998). In the case of other toxins, it has not been possible to induce entry from the cell surface by treating cells with low pH or other conditions, and it is therefore likely that, in this case, the entry mechanism is more complicated. The exception is CyaA from *B. pertussis* which appears normally to enter through the surface membrane (Rogel and Hanski 1992).

Considerable evidence has accumulated that endocytosis is required for the toxins to enter the cytosol. Thus, when endocytosis was blocked by depleting the cells of ATP, they were not able to take up the toxin (Sandvig and Olsnes 1982a). Other experiments indicating this phenomenon were carried out by depleting cells of calcium (Sandvig and Olsnes 1982b) or exposing them to slightly reduced pH, conditions that do not interfere with endocytosis. Under these conditions, the cells are still able to endocytose the toxins, but the toxins are not able to intoxicate the cells. Treatment with antibodies to inactivate toxins remaining at the cell surface and in the medium was then followed by incubation of the cells in normal medium to relieve the block. Under these conditions, the cells were intoxicated by toxin endocytosed during the block.

At temperatures below 18°C, endocytosis is still active, but transport to the trans-Golgi and to late endosomes is blocked. At this temperature, the cells were not intoxicated by ricin. The cells were, however, fully sensitive to diphtheria toxin under these conditions (Sandvig et al. 1984). Evidence has accumulated over time that transport to the trans-Golgi network is an important route for many toxins. Further experiments showed that brefeldin A and ilimaquinone, which lead to disintegration of the Golgi apparatus which is transported back to the ER, protect against ricin and Shigella toxin (Yoshida et al. 1991; Nambiar and Wu 1995; Rapak et al. 1997). These drugs did not affect the toxicity of diphtheria toxin. Clearly, therefore, there is a difference in the entry mechanism of the two groups of toxins. However, a main conclusion from these data is that, in all cases, endocytic uptake is a prerequisite for entry to the cytosol.

When the uptake of ricin by endocytosis from coated pits was blocked by depleting the cells of potassium, the cells were still intoxicated by ricin at approximately the same rate as in control cells. This provided the first evidence

that toxins can enter the cytosol by mechanisms bypassing endocytosis from coated pits. Later experiments showed that, when endocytic uptake from coated pits was inhibited by other mechanisms, such as acidification of the cytosol (but not of the medium) and by exposing the cells to hyperosmolaric medium, the cells were also intoxicated by ricin, but not by diphtheria toxin (SANDVIG et al. 1989).

There is now considerable evidence that several toxins are endocytosed from structures different from the coated pits. It appears, however, that the toxins endocytosed by such alternative pathways eventually end up in the same endosomes as those taken up by the classical, clathrin-dependent pathway (VAN DEURS et al. 1989).

D. Retrograde Vesicular Transport

I. Transport to the Golgi Apparatus

As first observed with cholera toxin, a certain fraction of the endocytosed toxin is transported to the trans-Golgi network (JOSEPH et al. 1979). Later experiments demonstrated that this is also the case in a number of other toxins, such as Shiga toxin and ricin (SANDVIG et al. 1992; RAPAK et al. 1997). Furthermore, in the case of ricin, it can be shown that the toxin is localized in the same compartment as newly synthesized export proteins. Thus, a cell line producing a monoclonal antibody against ricin was highly resistant to the toxin under conditions where the antibody was not able to inactivate the toxin at the cell exterior (YOULE and COLOMBATTI 1987). More direct evidence was obtained in ultrastructural studies demonstrating that ricin imported from the medium meets newly synthesized virus proteins in the trans-Golgi network (VAN DEURS et al. 1988). From there, it is then sorted and moved to various locations in the cells, such as the lysosomes or back to the cell surface. In polarized cells, the toxin may be transported to the opposite poles of the cells.

II. Transport to the Endoplasmic Reticulum

A most interesting recent development in studies of toxin transport is the observation that a considerable portion of the toxins that reach the Golgi apparatus are transported retrograde to the endoplasmic reticulum (ER). The first suspicion that this may be the case came from structural observations with cholera toxin and the related *E. coli* heat-labile toxin. Both these toxins consist of a pentameric B subunit that binds the toxins to gangliosides at the cell surface. The enzymatically active subunit, the A subunit, contains in its C-terminal end the sequence KDEL (cholera toxin) and RDEL (heat-labile toxin). Since the only known function of this sequence is to bind to the KDEL receptor, it was suspected that this sequence may indeed be involved in the intracellular routing of these two toxins (CHAUDHARY et al. 1990). Resident luminal ER proteins contain a C-terminal KDEL sequence. In the ER, where

the pH is neutral, the proteins are not bound to the KDEL receptor but, under the slightly acidic conditions prevailing in the Golgi apparatus, the sequence will become bound to the receptor, which will then transport the proteins back to the ER, where they are released. One function of the KDEL receptor, therefore, is to retrieve ER luminal resident proteins that have escaped to the Golgi complex. Evidently, an external protein that contains this sequence might also be transported to the ER if it has first reached the Golgi apparatus.

P. aeruginosa exotoxin A contains a C-terminal sequence that resembles the KDEL sequence, namely REDLK. Experiments have shown that this sequence, as such, does not bind to the KDEL receptor; however, it binds if the C-terminal lysine is first removed by cellular exopeptidases (although not with high affinity). It was proposed that this sequence may direct the toxin to the ER (Chaudhary et al. 1990). In fact, removal of the REDLK sequence abolished the toxicity of the molecule, while replacing it with the sequence KDEL resulted in recovery of the toxicity. This strongly indicates that transport to the ER is necessary for this toxin to act. However, it has not been formally demonstrated that exotoxin A is really transported to the ER.

The first convincing evidence for transport of an external protein retrograde to the ER came with another toxin, Shiga toxin (Sandvig et al. 1992). This toxin has a structure that resembles that of cholera and heat-labile toxin, but it does not have a KDEL-related sequence. Also, this toxin binds to a glycolipid, and it has been known for some time that it is transported to the trans-Golgi region and possibly to the Golgi stacks. Treatment with butyric acid was found to sensitize some cells to the toxin and, upon analysis of butyric-acid-treated cells that had been incubated with a conjugate of Shiga toxin and horseradish peroxidase, the conjugate was found in the ER, including the perinuclear space (Sandvig et al. 1992). Interestingly, the retrograde transport of Shiga toxin and cholera toxin was not inhibited by treatment with concanamycin, which blocks the proton pump in intracellular vesicles, whereas the retrograde transport of furin and TGN38 was inhibited under these conditions (Schapiro et al. 1998)

E. Translocation to the Cytosol

I. From the Surface

So far, only one bacterial toxin with an intracellular site of action has been demonstrated to be translocated from the cell surface, namely CyaA from *B. pertussis*. This protein (177 kDa) binds to, and apparently inserts into, membranes even at 4°C, as it cannot be removed by an alkaline carbonate wash. When the temperature is raised to 20°C or above, the toxin appears to translocate the enzymatically active part across the membrane. Calcium in the extracellular medium and an intact membrane potential are required for this process. Thus, when the cells were electrically depolarized, translocation did not occur. This raises the possibility that the enzymatically active part of the

molecule is electrophoresed across the membrane. A 45-kDa fragment carrying the enzymatic activity may subsequently be released into the cytosol (Rogel and Hanski 1992). Binding of calmodulin is required to activate the enzyme.

II. From Endosomes

Translocation from endosomes has been well characterized in two toxins, diphtheria toxin and anthrax toxin. In spite of their differences in primary structure and subunit composition, these two toxins are translocated by very similar mechanisms. Both toxins appear to be equipped with their own translocation devise. In the case of diphtheria toxin, this is part of the B fragment, which is also involved in binding to receptors. The B fragment contains a highly helical domain, the transmembrane or T domain. At low pH, as obtained in endosomes, this part of the toxin partially unfolds and inserts itself into the membrane, forming an ion-conducting pore initiated by a hairpin of hydrophobic helices (Kaul et al. 1996). The enzymatically active A-fragment is concomitantly translocated to the cytosol (Fig. 2).

Anthrax toxin belongs to the group of binary toxins. One part, the PA, binds to receptors at the cell surface and is then cleaved to release a 20-kDa fragment (Petosa et al. 1997). The rest of the molecule forms heptamers at the level of the cell surface and, at the same time, it generates a binding site for the other part of the toxin. This may be the edema factor or the lethal factor, which binds in a mutually exclusive manner. The heptamer formed by the cleaved PA inserts into the membrane at low pH and forms ion-conducting channels in the membrane in a manner very similar to that of diphtheria toxin. The enzymatically active part is translocated to the cytosol in the process (Fig. 3).

In the cases of both diphtheria toxin and anthrax toxin, it is necessary that the enzymatically active part is able to unfold during translocation. Thus, when disulfide loops are introduced into the A fragment of diphtheria toxin (it normally does not contain any), the translocation is blocked (Falnes et al. 1994). This is also the case if a ligand that confers tight folding is bound to a passenger protein that can otherwise be translocated along with the toxin (Wiedlocha et al. 1992). In the case of anthrax toxin, a fusion protein of diphtheria-toxin A fragment and the N-terminal part of anthrax-toxin lethal factor can be translocated into the cytosol, but the translocation is blocked by the introduction of a disulfide bridge in the molecule (Arora and Leppla 1994; Wesche et al. 1998).

III. From the ER

It now appears that an increasing number of toxins are translocated from the ER. Although several toxins have been demonstrated to be transported retrograde to the ER, it has been more difficult to demonstrate that the toxins

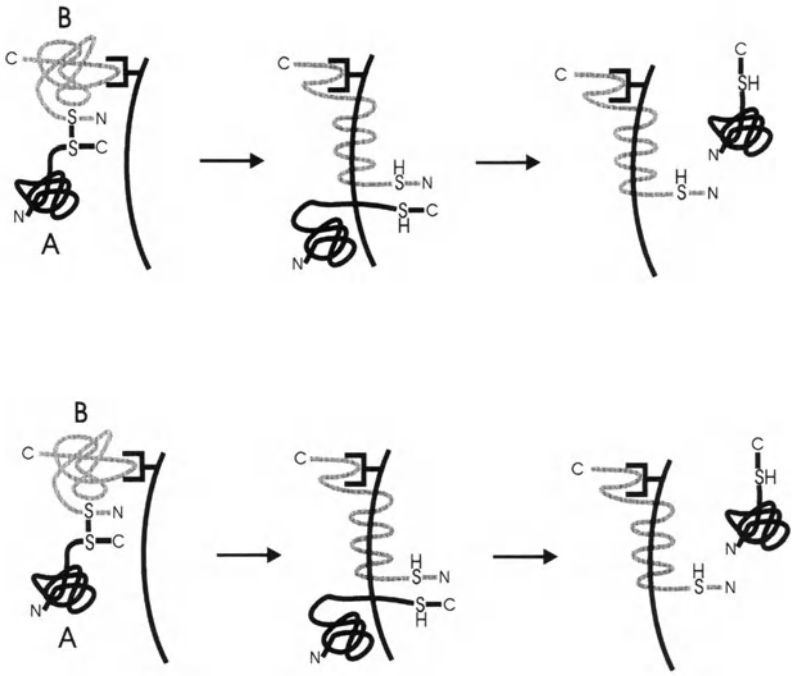

Fig. 2. Current model of translocation of diphtheria toxin. The toxin binds by its B fragment to cell-surface receptors and is endocytosed. Upon exposure to the low pH in endosomes, the helical T domain is exposed and inserts into the membrane, pulling the C-terminal end of the A fragment across the membrane. The disulfide bond is reduced upon exposure to the reducing conditions in the cytosol, and the remaining part of the A fragment is translocated to the cytosol

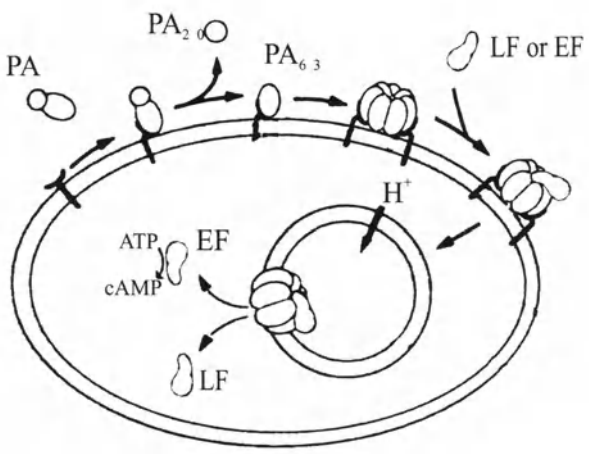

Fig. 3. Translocation of anthrax toxin. The protective antigen (*PA*) binds to cell-surface receptors, and an approximate 20-kDa piece is cleaved off. The remaining part of the molecule aggregates with other cleaved PA molecules to form a heptamer, thereby generating binding sites for lethal factor (*LF*) and edema factor (*EF*). The complex is endocytosed. Upon acidification of the endosome, the heptamer inserts into the vesicle membrane and facilitates translocation of LF and EF to the cytosol (Petosa et al. 1997)

are also translocated from this organelle. Certain plant toxins are very similar in structure and mechanism of action to bacterial toxins. One such plant toxin, ricin, acts on ribosomes in the same way as Shiga toxin, but it has a different B subunit; this subunit has lectin properties and binds to surface glycoproteins and glycolipids having terminal galactose residues (Olsnes et al. 1974). This toxin was the first to be shown to be transported retrograde, from the cell surface to the trans-Golgi network (Van Deurs et al. 1986, 1988) and, since sulfation occurs in the Golgi apparatus, we engineered a sulfation site into the C-terminal end of the toxin A chain. By subsequently incubating the cells with labeled sulfate, we could selectively label those molecules that had been transported to the Golgi apparatus. In other experiments, where we also engineered glycosylation sites into the protein, we could demonstrate that the labeled protein was partly glycosylated and that parts of the A-chain molecules could be recovered from the cytosol (Rapak et al. 1997). Importantly, it was mainly the glycosylated form that was found in the cytosol, indicating that only molecules that had been in the ER were translocated. Therefore, it is likely that the ER is the site of translocation.

This conclusion has become even more likely since it was found that misfolded export proteins are translocated retrograde, from the ER to the cytosol, where they become degraded by proteasomes (Hiller et al. 1996; Knop et al. 1996; Werner et al. 1996). A membrane-associated ubiquitinating enzyme appears to be important for this process. It is conceivable that toxins like ricin utilize this transport mechanism to penetrate the ER membrane. If that is so, the toxin must somehow avoid degradation by the proteasomes.

F. Stability of Toxins in the Cytosol

When wild-type diphtheria-toxin A fragment was microinjected into cells, it proved to be exceptionally stable. The natural A fragment has an N-terminal glycine residue, which is stabilizing, according to the N-end rule. However, even placing a destabilizing amino acid at the N-terminus of the A fragment did not result in very rapid degradation. However, when a Flag peptide was fused to the N-terminus, the N-terminal amino acid became very important for stability (Falnes and Olsnes 1998). When, in this case, a destabilizing amino acid was placed N-terminally, the toxic effect of the molecule was strongly reduced.

A survey of A chains that are translocated to the cytosol revealed that there was not a strong tendency to avoid destabilizing amino acids at the N-terminal end (Table 1). A possible reason for this is that a lysine residue, which could serve as a target for ubiquitination, is not present at a favorable distance from the N-terminus. In fact, it appears that toxins that may be translocated from the ER and might therefore be exposed to the membrane-bound ubiquitinating enzyme contain very few lysine residues; however, diphtheria toxin, which is translocated from endosomes and is therefore possibly less exposed

Table 1. The N-terminal residues of toxic proteins that enter the cytosol. The table lists experimentally determined or putative amino acids at the N-termini of the parts of protein toxins that are considered to enter the cytosol

Toxin	N-terminal amino acid
Diphtheria toxin, A chain	Gly[c]
Pertussis toxin, S1 subunit	Asp[i]
Ricin, A chain	Ile[h,n]
Abrin, A chain	Glu/Gln[l]
Tetanus toxin, light chain	Pro[f]
Botulinum neurotoxins (A, B, C1, E), light chain	Pro[g]
Botulinum neurotoxin D, light chain	Thr[g]
Shiga/Shiga-like toxin, A chain	Lys[b,k]
Cholera toxin, A chain	Asn[m]
Pseudomonas exotoxin, cytosolic fragment	Gly[j]
Mistletoe lectin I, A chain	Tyr[e]
Anthrax toxin, edema factor	Glu[d]
Anthrax toxin, lethal factor	Ala[a]

[a] Bragg and Robertson 1989.
[b] Calderwood et al. 1987.
[c] DeLange et al. 1979.
[d] Escuyer et al. 1988.
[e] Huguet-Soler et al. 1996.
[f] Krieglstein et al. 1991.
[g] Kurazono et al. 1992.
[h] Lamb et al. 1985.
[i] Nicosia et al. 1986.
[j] Ogata et al. 1990.
[k] Strockbine et al. 1988.
[l] Wood et al. 1991.
[m] Yamamoto et al. 1987.
[n] Funatsu et al. 1973.

to ubiquitinylation, contains a normal number of lysines (Hazes and Read 1997).

We found that ricin translocated to the cytosol accumulates to a larger extent in the presence of lactacystin (which inhibits the proteasomes) than in its absence. Therefore, some degradation of the translocated ricin apparently takes place in the absence of the inhibitor. In accordance with this, the cells were also somewhat sensitized to ricin in the presence of lactacystin.

G. Translocation of Fusion Proteins

It is an old concept that antibodies could be used to target toxins to cells of interest, such as cancer cells (Ehrlich 1957). Originally, it was thought that it is sufficient that the antibody be efficient in targeting the molecule to the surface of the target cells. From our present knowledge, this is only one criterion.

Immunotoxins may consist of an antibody linked to a whole toxin or only to its enzymatically active effector part. In the first case, the selectivity is usually low, as the conjugate will bind to the toxin receptors in addition to the target of the antibody. Higher selectivity is often obtained by linking only the effector moiety of the toxin to the antibody (OLSNES et al. 1989). However, in this case, the conjugate does not have a translocation moiety, and it is dependent upon the translocation ability of the target cells. It is likely that, in this case, the conjugate must be transported to the ER for translocation to take place. This means that the antibody must be directed against a cell-surface molecule that is able to direct the conjugate retrograde to the ER. So far, no such protein has been described whereas, in the case of lipids, such transport appears to take place. A number of natural toxins are, in fact, bound to cell-surface lipids.

In the future design of immunotoxins, it will be advisable to look for molecules that are able to route the conjugate retrograde, or to cross-link the receptor to another molecule that is able to direct the whole complex to the ER. A chimeric antibody reacting with both a cell-specific antigen and a molecule that is targeted to the ER could be able to carry out this task.

A more recent development in the field of toxin applications is to use toxins or their mutated, non-toxic counterparts to carry peptides into the cytosol for presentation by major histocompatibility complex (MHC) class-I molecules (STENMARK et al. 1991). There is a continuos breakdown of proteins in the cytosol, and some of the peptides produced by the proteasomes in this process are transported into the ER by the transporter associated with antigen processing (TAP) transporters (RÖMISCH 1994) and become bound to newly synthesized MHC class-1 proteins. The complex, together with β2-microglobulin, is transported to the cell surface, where it is presented to cytotoxic lymphocytes, which recognize the complex if it contains a peptide not present in the normal complement of proteins. Foreign proteins that appear in the cytosol, such as viral proteins and mutated autologous proteins (such as those present in cancer) are also broken down and, when their peptides are presented at the cell surface, they are recognized as foreign, and the cell is destroyed before the virus can multiply or before the malignant cell can develop into a tumor. Exposure to a foreign peptide presented in this way leads to expansion of the relevant cytotoxic T-cell clone, i.e. an immunization effect.

It has been difficult to obtain a vaccination effect in the MHC class-1 system, because the protein in question must be transported to the cytosol to be chopped up by the proteasomes into suitable pieces to bind to the MHC class-1 molecules. The ability of protein toxins to enter the cytosol opened up the possibility of using toxins as carrier molecules to transport passenger peptides and proteins into the cytosol (STENMARK et al. 1991; WIEDLOCHA et al. 1992; KLINGENBERG and OLSNES 1996). The possibility of detoxifying the molecules without depriving them of their ability to be translocated could allow the development of a vaccine that does not have the potential

side effects inherent in the use of attenuated viruses and injection of nucleic acids.

In attempts to test the principle, various peptides that are known to be presented by MHC class-1 molecules were fused to the N-terminus of the A fragment of diphtheria toxin. After binding the recombinant toxins to cells, it was possible to induce translocation of the fusion proteins into the cytosol in good yield (Stenmark et al. 1991). Attempts to demonstrate presentation by class-1 molecules were, however, unsuccessful.

Later, it was shown (with anthrax toxin and *B. pertussis* CyaA) that peptides fused to the toxins are indeed able to induce a class-1 immune response in mice (Ballard et al. 1996, 1998; Saron et al. 1997). This indicates, but does not prove, that the toxins transported the peptides to the cystosol by the toxin pathway. As mentioned above, it now appears that proteins that are misfolded in the ER are translocated backwards into the cytosol, where they are degraded by the proteasomes. Clearly, therefore, if a fusion protein is transported retrograde to the ER, it may be transported into the cytosol either as a conjugate with the toxin or after partial proteolytic degradation that could take place either at the level of endosomes, in the Golgi apparatus or in the ER.

Whole proteins, such as acidic fibroblast growth factor (Wiedlocha et al. 1992), dihydrofolate reductase (Klingenberg and Olsnes 1996) and a second A fragment of diphtheria toxin (Madshus et al. 1992) can be translocated into cells by the toxin pathway. Unfortunately, most whole proteins that were fused to the toxin were not translocated. Apparently, the reason for this is that the proteins must be able to unfold at low pH to follow the A fragment across the membrane, and most proteins are not able to do this.

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CHAPTER 2

Common Features of ADP–Ribosyltransferases

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A. Introduction

Mono-adenosine diphosphate (ADP) ribosylation is a post-translational modification of proteins that occurs in viruses, bacteria and eukaryotic cells (UEDA and HAYAISHI 1985; ALTHAUS and RICHTER 1987). During this reaction, the ADP–ribose moiety of nicotinamide adenine dinucleotide (NAD) is transferred onto an acceptor amino acid of the substrate molecule, which is usually forced to undergo a functional change (Fig. 1). When the reaction is mediated by a toxin, this event ultimately results in either malfunction or death of the target eukaryotic cells.

Based on their overall structure, ADP–ribosylating toxins can be divided into A/B toxins, binary toxins and A-only toxins, depending on whether they have the catalytic and binding domains in the same molecule, in different molecules, or whether the binding domain is totally absent (Table 1). In all cases, the A domain bears the catalytic site and is thus responsible for the toxic activity, while the B subunit, when present, appears to be involved in recognition and binding of the whole molecule to the specific surface receptor. The B subunit is also involved in the translocation of the toxic moiety within the cell (MIDDLEBROOK and DORLAND 1984).

B. The Well-Characterized Toxins

Among mono-ADP–ribosyltransferases, the bacterial toxins, such as cholera toxin (CT; MEKALANOS et al. 1983), heat-labile enterotoxin from *Escherichia coli* (LT; SPICER and NOBLE 1982; YAMAMOTO et al. 1984), pertussis toxin (PT; LOCHT et al. 1986; NICOSIA et al. 1986), diphtheria toxin (DT; PAPPENHEIMER 1977; COLLIER 1982) and *Pseudomonas aeruginosa* exotoxin A (PAETA; GRAY et al. 1984; WICK et al. 1990), are the best characterized in terms of molecular structure and substrate specificity. Functional and structural studies performed on these toxins allowed the understanding of the general rules, which can be extended to all the ADP–ribosylating enzymes known to date. PT, CT and LT are typical examples of the A/B family of toxins (Table 1), where the two subunits are linked together by non-covalent bonds.

Table 1. List and main properties of known adenosine diphosphate (ADP)–ribosylating enzymes

Enzyme	Organism	Structure	Acceptor protein	Acceptor residue	Effect on eukaryotic cells	X-ray structure	Reference
CT	*Vibrio cholerae*	A/B	G_s, G_{olf}, G_t	Arg201	Alteration of transmembrane-signal transduction	Yes	Mekalanos et al. 1983
LT	*Escherichia coli*	A/B	G_s, G_{olf}, G_t	Arg201	Alteration of transmembrane-signal transduction	Yes	Spicer and Noble 1982
LT-II	*E. coli*	A/B	G_s, G_{olf}, G_t	Arg201	Alteration of transmembrane-signal transduction	No	Pickett et al. 1987
PT	*Bordetella pertussis*	A/B	G_i, G_o, G_{gust}, G_t	Cys352	Alteration of transmembrane-signal transduction	Yes	Locht et al. 1986
DT	*Corynebacterium diphtheriae*	A/B	EF-2	Diphthamide 715	Inhibition of protein synthesis	Yes	Collier 1982
PAETA	*Pseudomonas aeruginosa*	A/B	EF-2	Diphthamide 715	Inhibition of protein synthesis	Yes	Gray et al. 1984
MTX	*Bacillus sphaericus*	Binary	Not known	Not known	Toxicity to mosquito larvae	No	Thanabalu et al. 1991
ExoS	*P. aeruginosa*	A only	Ras	Asn41	Inhibition of actin polymerization	No	Kulich et al. 1994
C3	*Clostridium botulinum*	A only (?)	Rho	Asn41	Disruption of actin cytoskeleton	No	Nemoto et al. 1991
C2	*C. botulinum*	Binary	Actin	Arg177	Inhibition of actin polymerization	No	Barth et al. 1998
IOTA_tox	*Clostridium perfringens*	Binary	Actin	Arg177	Inhibition of actin polymerization	No	Perelle et al. 1995
C3	*Clostridium limosum*	A only (?)	Rho	Asn41	Disruption of actin cytoskeleton	No	Just et al. 1992
Iota-like toxin	*Clostridium spiroforme*	Binary	G-actin	Arg177	Inhibition of actin polymerization	No	Popoff and Boquet 1988a
CDT	*Clostridium difficile*	Binary	G-actin	Arg177	Inhibition of actin polymerization	No	Popoff and Boquet 1988b
Exoenzyme	*Bacillus cereus*	A only (?)	Rho	ASN41	Disruption of actin cytoskeleton	No	Just et al. 1995
EDIN	*Staphylococcus aureus*	A only (?)	Rho	ASN41	Disruption of actin cytoskeleton	No	Sugai et al. 1990

DraT	*Rhodospirillum rubrum*	?	Dinitrogenase reductase	Not known	Not known	No	FITZMAURICE et al. 1989
Halovibrin	*Vibrio fischeri*	?	Not known	Not known	Not known	No	REICH et al. 1996
T4	Bacteriophage T4	?	RNA polymerase	*C*-terminus	Increase of transcription	No	WILKENS et al. 1997
T2	Bacteriophage T2	?	RNA polymerase	*C*-terminus	Increase of transcription	No	KOCH and RUGER 1994
RT6-1	Mouse	?	Arginine-rich histones, M2 antibody[a]	Arg	Not known	No	KOCH-NOLTE et al. 1996
RT6-2	Mouse	?	Arginine-rich histones, M2 antibody[a]	Arg	Not known	No	KOCH-NOLTE et al. 1996
RT6.1	Rat	?	Not known	Arg	Not known	No	HAAG et al. 1990
Yac-1	Mouse	?	Not known	Arg	Regulation of proliferation and cytotoxicity	No	OKAZAKI et al. 1996b
Yac-2	Mouse	?	Agmatin[a], guanidino compounds	Arg	Prevalent NAD-glycohydrolase activity	No	OKAZAKI et al. 1996a
PARP	Human	?	Core histones (H2B), linker histone (HI) and others	Glu	Inhibition of DNA repair and increase of DNA damage	No	MARSISCHKY et al. 1995
	Mouse	?	Core histones (H2B), linker histone (HI) and others	Glu	Inhibition of DNA repair and increase of DNA damage	No	HUPPI et al. 1989
	Chicken	?	Core histones (H2B), linker histone (HI) and others	Glu	Inhibition of DNA repair and increase of DNA damage	Yes	RUF et al. 1996
Tankyrase	Human	?	TRF1, tankyrase	TRF1 *N*-terminus	Increase of telomere length	No	SMITH et al. 1998

CT, cholera toxin; *DT*, diphtheria toxin; *EDIN*, epidermal differentiation inhibitor; *EF*, elongation factor; *ExoS*, exoenzyme S; *LT*, heat-labile enterotoxin; *MTX*, mosquitocidal toxin; NAD, nicotinamide adenine dinucleotide; *PAETA*, *Pseudomonas aeruginosa* exotoxin A; *PARP*, poly-ADP–ribosyl polymerases; *PT*, pertussis toxin.
[a]Non-physiological target proteins.

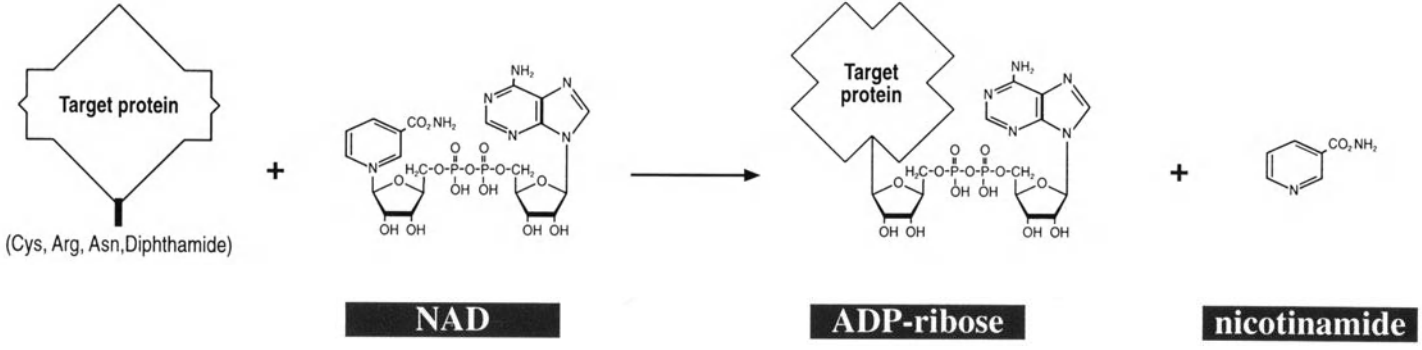


Fig. 1. Mechanism of the mono-adenosine diphosphate (ADP)–ribosylation catalyzed by ADP–ribosyltransferases. An ADP–ribose moiety is transferred to a specific target protein, and a nicotinamide group is released

In the case of PT, the A domain is represented by the subunit S1 (21 kDa), which bears the enzymatic core; S1 is also responsible for the binding of NAD and for the transfer of the ribose group to an acceptor amino acid (cysteine) of a group of guanosine triphosphate (GTP)-binding proteins, such as G_i and G_o (KATADA et al. 1983; WEST et al. 1985), that are involved in the mechanism of signal transduction in eukaryotic cells. B is a non-toxic oligomer formed by four distinct subunits named S2 (22 kDa), S3 (21.8 kDa), S4 (12 kDa) and S5 (11.7 kDa), where S4 is present in two copies (TAMURA et al. 1982). The B oligomer binds the receptor on the surfaces of eukaryotic cells and facilitates the translocation of the enzymatically active portion across the cell membrane, where it can reach the target G proteins.

In the case of CT and LT, the main target for the ADP–ribosylation reaction promoted by the A subunit (27 kDa) is Gs, a protein which activates the adenylate cyclase, thus inducing the synthesis of the cyclic adenosine monophosphate second messenger (GILMAN 1984). The B oligomer is formed by five identical subunits (11.7 kDa each) that assemble into a pentameric structure and are together responsible for the binding to the receptor (Moss and VAUGHAN 1988). The genes coding for CT and LT are highly homologous (DALLAS and FALKOW 1980; SPICER et al. 1981; DOMENIGHINI et al. 1995) and are organized into operons located on the chromosome of *Vibrio cholerae* and on a plasmid of *E. coli* (So et al. 1978), respectively.

Slightly different are the architectures of DT (58.3 kDa) and PAETA (67 kDa), which are nevertheless included in the class of A/B toxins (Table 1). They are composed of three domains, of which the catalytic domain C is contained in fragment A, while the transmembrane domain T and the receptor-binding domain R are contained in fragment B. Both of them display their toxic activity by transferring the ADP–ribose moiety to a post-translationally modified histidine residue of eukaryotic elongation factor 2 (EF-2; BROWN and BODLEY 1979; NESS et al. 1980), thus blocking protein synthesis and killing the cell.

With the exceptions of CT and LT, which share more than 80% amino acid identity (DALLAS and FALKOW 1980; SPICER et al. 1981; DOMENIGHINI et al. 1995), no structural similarities can be detected among the various B domains. This observation is generally justified by the different specificity the toxins display for their eukaryotic receptors. Also, in the case of the A subunits, no significant and extended sequence homology that was able to justify the observed common mechanism of catalysis could be detected.

Nevertheless, biochemical experiments on photoaffinity labeling and studies of site-directed mutagenesis demonstrated that all the toxins possess a catalytic glutamic acid so important for enzymatic activity that not even a conservative substitution with an aspartic acid can be tolerated without loss or drastic decrease of toxicity (DOUGLAS and COLLIER 1987; WILSON et al. 1990; LOBET et al. 1991; ANTOINE et al. 1993). This finding suggested that the active sites might display more structural similarities than those anticipated by the lack of a clear sequence homology and prompted a number of studies that

finally led to the identification of a common architecture for the enzymatic cores of most bacterial ADP–ribosyltransferases (DOMENIGHINI et al. 1994; DOMENIGHINI and RAPPUOLI 1996).

C. A Common Structure for the Catalytic Site

Today, crystallographic data are available for LT (SIXMA et al. 1991), CT (ZHANG et al. 1995), PT (STEIN et al. 1994), DT (CHOE et al. 1992) and PAETA (ALLURED et al. 1986). Computer-modeling studies based on the comparative analysis of the above-mentioned X-ray structures helped to identify a minimal conserved region necessary for NAD binding and catalysis; this region was almost perfectly superimposable for all the bacterial toxins (DOMENIGHINI et al. 1994).

In terms of tertiary structure, the active site is formed by a β-strand followed by a slanted α-helix, which has a different length in the various toxins (spanning from 12 residues for DT, PAETA and LT, to 21 residues in the case of PT). The β-strand and the α-helix represent, respectively, the lower and the upper faces of the cavity in which the nicotinamide ring of NAD enters and is anchored during the enzymatic reaction (Fig. 2).

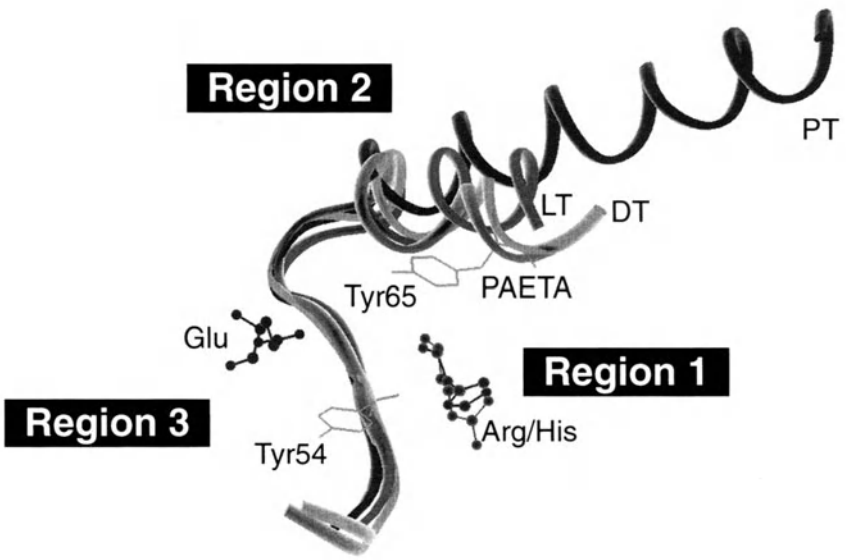


Fig. 2. Superimposition of the three-dimensional structures of the nicotinamide adenine dinucleotide-binding cavities (region 2) of the bacterial toxins heat-labile enterotoxin (LT), pertussis toxin (PT), diphtheria toxin (DT) and *Pseudomonas aeruginosa* exotoxin A (PAETA). The catalytic residues brought by region 1 (Arg/His) and by region 3 (Glu) and common to the two homologous groups (Fig. 3) are shown. In addition, the two essential tyrosines of the DT group are indicated. Primary structures of region 1, 2 and 3 (and their alignments) are reported in Fig. 3

A careful analysis of the primary structure of the A subunits showed that they can be classified into two groups: the DT-like group, mainly composed of DT, PAETA and human poly-ADP–ribosyl polymerases (PARPs), and the CT-like group, comprising CT, LT, PT, the mosquitocidal toxin (SSII-1) of *Bacillus sphaericus*, exoenzyme S (ExoS) of *P. aeruginosa*, iota toxin of *Clostridium perfringens* and other bacterial and eukaryotic ADP–ribosyltransferases (Fig. 3). Although all the toxins share a similar tertiary structure in the region of the catalytic domain, at the amino acidic level the only residue which is strictly conserved in all the toxins of the DT and CT subgroups is the glutamic acid mentioned above (namely Glu148 of DT, Glu 553 of PAETA, Glu112 of CT and LT and Glu129 of PT). The above residues retain an equivalent spatial position and orientation, residing in a short β-strand flanking the external side of the cavity.

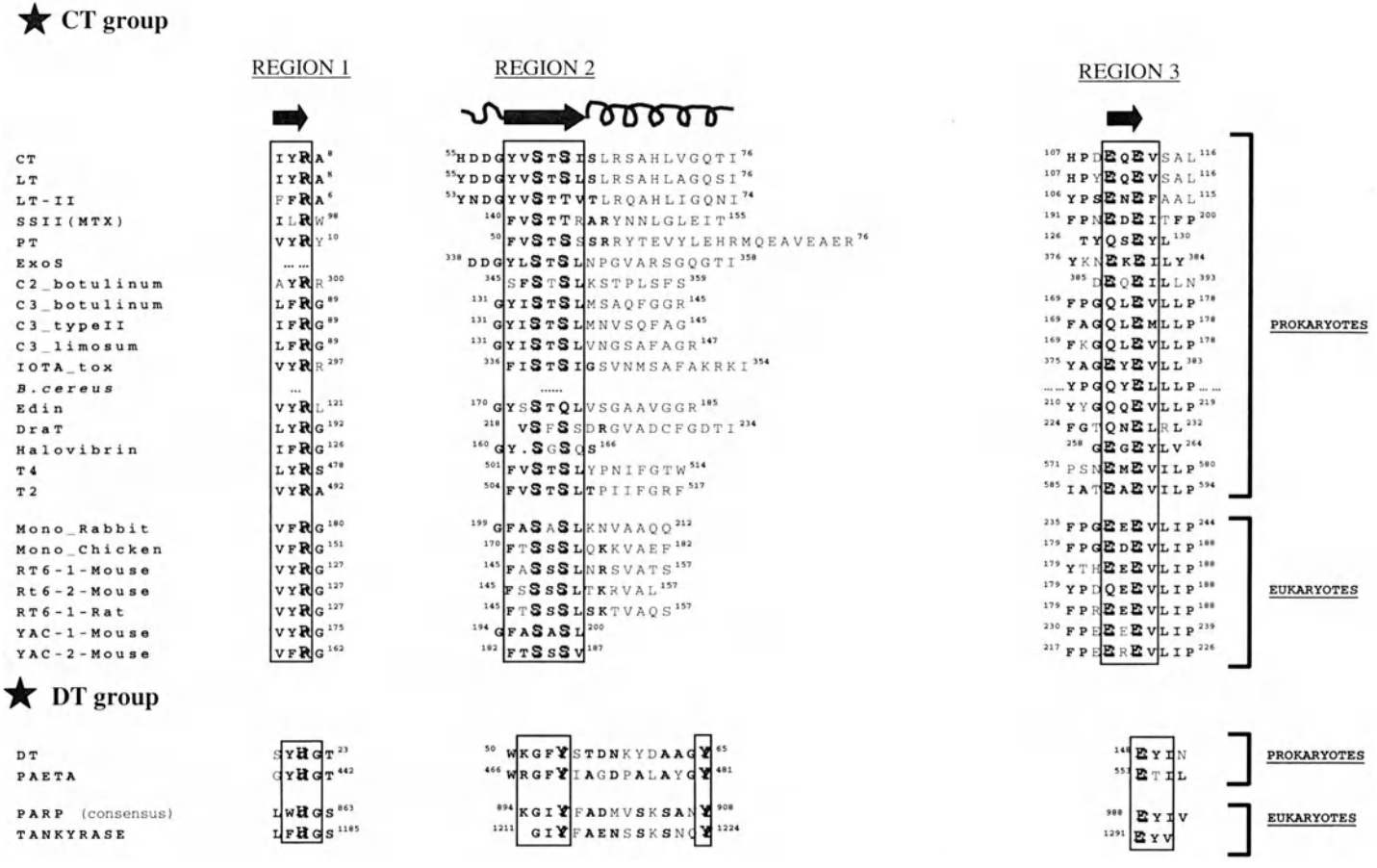
Another residue that is well conserved along the sequences is His21 of DT. This residue can be aligned to His440 of PAETA and to Arg7 and Arg9 of CT–LT and PT, respectively. The available crystal structures reveal that these amino acids are located in essentially identical positions within the active site cleft; they are opposite the glutamic acid on an antiparallel β-strand close to the internal face of the catalytic site. Experiments using site-directed mutagenesis involving His21 of DT, His440 of PAETA, Arg9 of PT or Arg7 of CT and of LT diminished or completely abolished ADP–ribosylation activity, thus suggesting a functional relationship between these residues and a common important role in the enzymatic activity (Burnette et al. 1988, 1991; Papini et al. 1990; Lobet et al. 1991; Han and Galloway 1995).

Although several models have been proposed to explain its possible function, it is now widely accepted that this residue does not play a direct role in catalysis; alternatively, it may have a function in maintaining the integrity of the active-site pocket upon formation of structurally stabilizing hydrogen bonds (Johnson and Nicholls 1994). In the case of His21 of DT, in fact, the only acceptable substitution is with glutamine, the only residue capable of maintaining two hydrogen bonds. Nevertheless, mutations at the His440 position of PAETA, while affecting the enzymatic activity, have little or no effect on NAD binding (Han and Galloway 1995). This suggests that His440 may not be exactly homologous to His21 of DT or to the arginines of the CT group.

Extending the analysis to all the primary structures known to date, we can delineate three major blocks of homology, referred to as regions 1, 2 and 3 (Fig. 3). Regions 1 and 3 contain the amino acids involved in catalysis, whereas region 2 delineates the scaffold of the cavity.

I. Region 1

Region 1 is characterized by the above-mentioned Arg/His residue and is well conserved between the DT and CT groups. This domain can be extended upstream to a couple of well-conserved amino acids, where the first is


CT group
REGION 1
REGION 2
REGION 3
CT
LT
LT-II
SSII(MTX)
PT
ExoS
C2_botulinum
C3_botulinum
C3_typeII
C3_limosum
IOTA_tox
B.cereus
Edin
DraT
Halovibrin
T4
T2
Mono_Rabbit
Mono_Chicken
RT6-1-Mouse
Rt6-2-Mouse
RT6-1-Rat
YAC-1-Mouse
YAC-2-Mouse
PROKARYOTES
EUKARYOTES
DT group
DT
PAETA
PARP (consensus)
TANKYRASE
PROKARYOTES
EUKARYOTES

hydrophobic (generally Leu or Val) and the second aromatic (generally Tyr or Phe; Fig. 3).

II. Region 3

The domain referred to as region 3 shows a different consensus pattern in the two groups of toxins. In the DT family, in fact, it is composed by the only catalytic glutamic acid (followed by an aromatic and a hydrophobic residue) while, in the CT group, the consensus can be extended to a few neighboring residues. In particular, a second polar amino acid, Glu or Gln, is always present upstream of the essential Glu, and a short stretch of hydrophobic residues follows downstream in most cases. X-ray structures of LT and PT show that the side-chains of these polar residues are parallel and extend towards the internal portion of the catalytic domain, thus suggesting a potentially equivalent role for them in the mechanism of catalysis.

The recent publication of crystallographic data for the DT–NAD complex (Bell and Eisenberg 1996) establishes that the carboxylate group of the side chain of Glu148 lies near the nicotinamide ring of NAD, at a distance of 4 Å

Fig. 3. Sequence alignment of protein segments containing regions 1, 2 and 3 of known adenosine diphosphate (ADP)–ribosylating enzymes. Two groups of homology are reported. The cholera toxin (CT) group comprises sequences from bacterial species, such as: CT (PIR = A05129), heat-labile enterotoxin (LT; SWISS = P06717), LT type II (PIR = A29831), *Bacillus sphaericus* mosquitocidal toxin SSII (PIR = S27514), *Bordetella pertussis* toxin (SWISS = P04977), *Pseudomonas aeruginosa* exoenzyme S (GenBank = L27629), *Clostridium botulinum* toxins (DDBJ = D88982), C3 (PIR = A41021) and C3 type II (SWISS = P15879), *C. limosum* exoenzyme C3 (EMBL = X87215), *C. perfringens* iota toxin (GenBank = X73562), *Bacillus cereus* exoenzyme (a fragment in NCBI gibbsq159913), *Staphylococcus aureus* epidermal differentiation inhibitor (PIR = JG0016), *Rhodospirillum rubrum* nitrogen-regulatory factor DraT (SWISS = P14299) and *Vibrio fischeri* halovibrin (GenBank = U38815). The CT group also features sequences from ADP–ribosyltransferases produced by bacteriophages T4 (SWISS = P12726) and T2 (GenBank = X69893), sequences from the eukaryotic mono-ADP–ribosyltransferases: mono-rabbit (SWISS = Q03515) and mono-chicken (EMBL = X82397), RT6-1 (SWISS = P17981) and RT6-2 (GenBank = AF016465) from mouse, the corresponding RT6-1 (SWISS = P17982) and RT6-2 (SWISS = P20974) from rat and Yac-1 (SWISS = Q60935) and Yac-2 (GenBank = U60881) from mouse. The diphtheria toxin (DT) group comprises sequences from the bacterial proteins *Corynebacterium diphtheriae* toxin DT (SWISS = P00588) and *P. aeruginosa* exotoxin A, PAETA (SWISS = P11439), and from the eukaryotic poly-ADP–ribosyl polymerases (PARPs; the consensus of nine sequences is reported, but the numbering refers to the human PARP, SWISS = P09874). The human RF1-interacting ankyrin-related ADP–ribose polymerase, Tankyrase, has been added (GenBank = AF082556). Catalytic residues of regions 1 and 3 and the most relevant and conserved residues in region 2 are reported as shaded letters. Extended consensus sequences detected in the three regions are *boxed*, while other partially conserved residues are in *bold*. In terms of secondary structure, regions 1 and 3 are observed and predicted to be folded as short β-strands (*arrow*) that face on opposite sides the enzymatic cavity corresponding to region 2. The folding of region 2 is characterized by a portion of unstructured coil (*solid line*) followed by a β-strand (*arrow*) and an α-helix. Numbering of the sequences is reported in *upper case*

from the *N*-glycosidic bond. Applying this observation to all the toxins, the more plausible explanation in terms of enzymatic activity is that the essential glutamic acid could play a role in stabilizing a chemical intermediate of NAD during a S_N1-type mechanism, in which the nicotinamide ring might be dissociated.

III. Region 2

The other reported block of homology is region 2, which includes a number of amino acids that, while maintaining the same secondary structure in both DT and CT families (Fig. 2), still strongly differ in terms of sequence (Fig. 3). This region corresponds to the core of the active-site cleft, which is devoted to the docking of NAD. The difference detected, in terms of primary structure, between the two subgroups suggests that the toxins may adopt diverse mechanisms of binding. The consensus sequence generated for the DT group is characterized by two conserved tyrosines spaced by ten amino acids and located on the middle portion of the β-strand and on the internal face of the α-helix, respectively (Fig. 2).

Tyr 54 and Tyr 65 of DT and Tyr 470 and Tyr 481 of PAETA have been shown to play a very important role in catalysis, since they anchor the nicotinamide ring, creating a π pile of three aromatic rings, which strengthens the overall binding of NAD and stabilizes the complex (CARROLL and COLLIER 1984; LI et al. 1995). In PT, a similar role is likely to be played by Tyr 59 and Tyr 63, which have a similar spatial orientation and distance from each other. This observation is supported by the fact that in CT and LT, where the stacking interactions produced by the two tyrosines are lacking, the affinity for NAD is 1000-fold lower (GALLOWAY and VAN HEYNINGEN 1987).

The consensus observed for the DT group can be extended upstream to another three residues (Arg/Lys–Gly–hydrophobic/aromatic) which precede the first tyrosine (Fig. 3). In the case of the CT group, region 2 is centered on a conserved core domain characterized by the consensus Ser–Thr–Ser, which is observed and predicted to fold into a β-strand representing the floor of the cavity. No conclusive information is available to define the precise role of these small, polar residues in catalysis, but experiments using site-directed mutagenesis confirm that they are extremely important in maintaining the cavity available for NAD entrance and docking.

Substitution of Ser 61 of LT with Phe has been shown to produce a nontoxic mutant (HARFORD et al. 1989). In a similar manner, LT and CT mutants where Ser63 is substituted by Lys (PIZZA et al. 1994; FONTANA et al. 1995) result in a complete loss of toxic activity while still maintaining a good capacity for the production of the holotoxin. This phenomenon can be explained by the analysis of the crystal structure of LT-Ser63Lys (LTK63) (VAN DER AKKER et al. 1997), which has recently been resolved.

The mutant, in fact, appears to be completely identical to the wild-type LT across the entire molecule, with the exception of the active site, where

Lys63 introduces into the cavity a bulky and charged side chain that, while filling the space usually occupied by NAD (thus impairing its binding; Fig. 4A) can, concurrently, stabilize the structure by forming a saline bridge with Glu112. On the basis of these data, the core region Ser–Thr–Ser can be extended to give the more general consensus "aromatic–hydrophobic–Ser–Thr–Ser–hydrophobic".

Another amino acid that has been proposed as being important in catalysis is His35 of PT (Xu et al. 1994). This is located near the beginning of the β-strand which forms the floor of the cavity, in a position equivalent to that of His44 of LT and CT (Yamashita et al. 1991). A functionally homologous His is also present in the mosquitocidal toxin SSII-1 from *B. sphaericus* (Thanabalu et al. 1991) but is absent in DT and PAETA. In the three-dimensional structure, this residue appears to be sufficiently close to the oxygen atom of

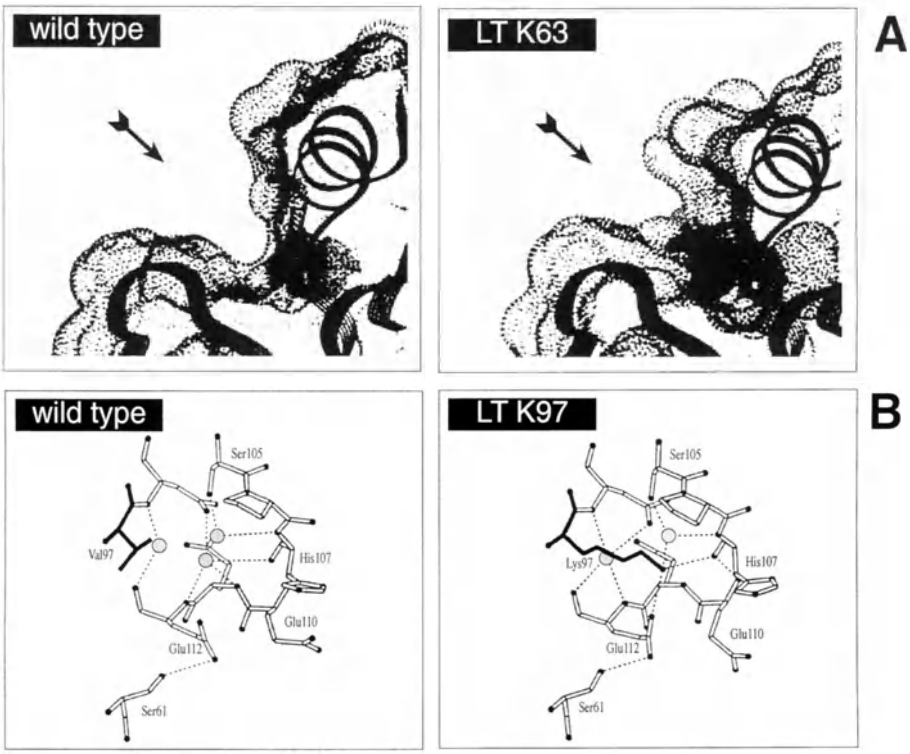


Fig. 4. Description of two of the most interesting mutants constructed for heat-labile enterotoxin (LT). **A** Three-dimensional structure of the enzymatic cavity of the wild type LT (*left*) and of the mutant LTK63 (*right*). The *arrows* point out how much this single point mutation can affect the dimensions of the pocket and, thus, the entrance of a nicotinamide adenine dinucleotide molecule. **B** Structure of the wild type LT (*left*) and of the mutant LTK97 (*right*). Glu112 is involved in one hydrogen bond in the native structure and in two in the mutant

the ribose ring of NAD to interact with it and increase the electrophilicity of the adjacent anomeric carbon atom. The absence of an equivalent residue in DT and PAETA again supports the idea that the two groups of toxins perform the same enzymatic activity in slightly different fashions.

An additional feature that is common to all ADP–ribosylating toxins is the need for a conformational rearrangement in order to achieve enzymatic activity. In the native structure, in fact, the NAD-binding sites of LT and CT are obstructed by a loop comprising amino acids 47–56; this loop needs to be displaced in order to produce a functional NAD-binding cavity. A functionally homologous region is also present in PT, where the loop comprises residues 199–207.

In the case of DT, where the crystallographic data of the complex are available, the observation that the active-site loop, consisting of amino acids 39–46, changes structure upon NAD-binding suggests that these residues may be important for the recognition of the ADP–ribose-acceptor substrate, EF-2 (WEISS et al. 1995; BELL and EISENBERG 1996). This proposal is supported by at least two lines of evidence. First, DT and PAETA have a high degree of sequence similarity in this loop region, with a number of identical or highly conservative substitutions, supporting the idea that these residues have some essential function (CARROLL and COLLIER 1988; OLSON 1993). Second, antibodies raised against a peptide corresponding to this loop sequence were able to prevent the catalytic domain of DT from catalyzing the ADP–ribosylation of EF-2 (OLSON 1993).

In conclusion, the reported studies of X-ray and sequence analysis, supported by experimental evidence, have clearly demonstrated that all the bacterial ADP–ribosylating toxins known to date share some basic features. In particular, they are characterized by a common folding of the NAD-binding site, by the existence of few conserved amino acids involved in catalysis, and by the requirement for a conformational change in order to acquire activity.

The recent publication of crystallographic data for the DT–NAD complex and the presence of common features within all ADP–ribosylating toxins allow us to speculate on a possible common mechanism of catalysis. The best hypothesis is that NAD enters the cavity upon displacement of the mobile loop, which is then made available for the recognition of the substrate. NAD is subsequently docked at the bottom of the pocket, where a small residue (the conserved serine in the β-α box of the CT group or Thr56 of DT and Ala472 of PAETA) is required to allow good positioning. The nicotinamide moiety of NAD is then blocked in a suitable position by means of stacking interactions provided by a couple of aromatic rings (Tyr 54 and Tyr 65 of DT, Tyr 470 and Tyr 481 of PAETA and possibly Tyr 59 and Tyr 63 of PT). In this context, the conserved Arg/His might display its key role in maintaining the correct shape of the active-site pocket via hydrogen bonds formed with the backbone of the structure and possibly with the ribose moiety. The enzymatic reaction is then catalyzed by the essential glutamic acid, which is likely to stabilize a positively charged oxocarbonium intermediate of NAD, in order to favor its subsequent

interaction with the nucleophilic residue of the incoming substrate (diphthamide in the cases of DT and PAETA, arginine in the cases of LT and CT, and cysteine in the case of PT).

D. Other Bacterial Toxins with ADP–Ribosylating Activity

In addition to the well-characterized ADP–ribosyltransferases described in the previous sections, a number of other bacterial toxins that share a similar mechanism of action and some degree of sequence similarity in the region of the active site have been identified. Among these, the most represented family is that of clostridial toxins (Aktories 1994), comprising *C. botulinum* C2 toxin (Aktories et al. 1986) and exoenzyme C3 (Nemoto et al. 1991), *C. perfringens* iota toxin (Perelle et al. 1995) and *C. spiroforme* and *C. difficile* ADP–ribosylating toxins (Popoff and Boquet 1988a, 1988b).

C. perfringens iota toxin, *C. spiroforme* and *C. difficile* ADP–ribosylating toxins have a structure and function very similar to those of C2 toxin of *C. botulinum*; they are all included in the class of binary toxins (Table 1). However, they are usually classified in a separate family of iota-like toxins, because they are immunologically related and do not show cross-reaction with the C2 toxin.

The structural gene of the enzyme component C2I of the binary toxin C2 of *C. botulinum* has recently been cloned and sequenced (Fujii et al. 1996). Although no significant sequence identity with any other transferase was reported, a homology of 33% with the enzyme component of iota toxin was detected. Based on the alignment of C2I with iota toxin and other ADP–ribosyltransferases, the key residues were identified and tested with experiments using site-directed mutagenesis (Barth et al. 1998; Fig. 3). Change of Glu389 to Gln blocked the ADP–ribosyltransferase activity and the NAD-glycohydrolase activity and prevented the cytotoxic effects of this toxin; thus, Glu389 of C2I appears to be functionally equivalent to Glu148 of DT, Glu554 of PAETA, Glu112 of LT and Glu129 of PT.

As in the case of most bacterial toxins, even the C2I component of *C. botulinum* possesses a second glutamic acid two residues upstream of the invariant catalytic glutamate. The exchange of this second glutamic acid residue (Glu387) to glutamine in C2I results in a loss of transferase activity; surprisingly, this mutant still hydrolyzed NAD, indicating that this residue is essential for ADP–ribosyltransferase activity but is not required for NAD-glycohydrolase activity. In a similar fashion, substitution of Arg299 with Ala induced a dramatic reduction in transferase activity; thus, this residue is the equivalent of Arg7 of LT, Arg9 of PT, His21 of DT and Arg295 of iota toxin (Perelle et al. 1996, Barth et al. 1998).

In the C2I toxin, the S–T–S motif central to region 2 is extended to S–S–T–S (Ser347–Ser350). Substitution of the first or the last serine residues

to alanine did not change the transferase activity substantially. Similarly, the change of both Ser347 and Ser 350 to alanines reduced but did not abolish transferase activity. These findings support the notion that neither residue Ser347 nor Ser350 are essential for catalysis. Whereas change of Thr349 to Val reduced the enzyme activity, the substitution of Ser348 with Ala eliminated transferase activity. Thus, Ser348 is very likely the correspondent of Ser61 of LT and may play an essential role in NAD binding or catalysis.

The exoenzyme C3 of *C. botulinum* is a 25-kDa ADP–ribosyltransferase, which apparently lacks the B moiety typical of the other toxins and, therefore, cannot enter eukaryotic cells. This toxin, which is therefore described as an A-only toxin (Table 1), is the prototype of a number of C3-like exoenzymes, such as *C. limosum* (JUST et al. 1992; JUNG et al. 1993) and *B. cereus* exoenzymes (JUST et al. 1995) and the epidermal differentiation inhibitor of *Staphylococcus aureus* (SUGAI et al. 1990). All these transferases have molecular masses of 25–28kDa, are rather basic proteins and appear to ADP–ribosylate GTP-binding proteins of the Rho family at an asparagine residue. Experiments using affinity labeling have identified Glu174 as the residue likely corresponding to the catalytic glutamic acid residue of bacterial ADP–ribosyltransferases. A glutamic acid residue conserved among the C3-like exoenzymes has also been identified (Fig. 3).

P. aeruginosa is an opportunistic pathogen of humans which produces both cell-associated and secreted virulence factors. In particular, it synthesizes two ADP–ribosyltransferases, namely PAETA, which we have already described, and ExoS (KULICH et al. 1994), which was purified from the culture supernatant in both 53- and 49-kDa forms, with only the latter showing enzymatic activity.

ExoS lacks both the receptor-binding and the translocation domains; therefore, it belongs to the group of the A-only toxins (Table 1). In this case, bacteria intoxicate individual target cells by using a contact-dependent secretion system to inject or deliver toxic proteins into the cytoplasm of eukaryotic cells; in particular, ExoS is the only ADP–ribosylating toxin that has been shown to be delivered by a type III secretion system (YAHR et al. 1996).

Studies of sequence homology have identified short regions of primary amino acid similarity between ExoS and members of the CT-like group of bacterial toxins. In particular, studies of site-directed mutagenesis show that the catalytic Glu112 of LT and Glu148 of DT can be aligned with Glu381 of ExoS (LIU et al. 1996), while the conserved S–T–S motif has an equivalent region in ExoS at positions 343–345.

Another toxin included in Fig. 3 is the 100-kDa mosquitocidal toxin SSII-1 isolated from strains of *B. sphaericus*. The primary sequence of SSII-1 was compared with those of known toxins, and some regional homology was detected with bacterial ADP–ribosyltransferases (THANABALU et al. 1991).

In particular, Arg97 of SSII-1 aligns with Arg9 of PT and Arg7 of CT and LT, and the region spanning amino acids 140–144 (F–V–S–T–T) appears to be well conserved with region 2 of the CT-like family of toxins. On the basis of

these observations, the possibility that *B. sphaericus* SSII-1 toxin might have ADP–ribosyltransferase activity is under investigation.

Finally, the purification, cloning and deduced amino acid sequence of a member of a new class of ADP–ribosyltransferases was reported by Reich and Schoolnik in 1996. This enzyme, named halovibrin, was purified from the culture supernatant of the marine bacterium *V. fischeri*.

Recombinant *E. coli* clones harboring the DNA fragment containing the gene for halovibrin were shown to possess ADP–ribosyltransferase activity. Analysis of the deduced amino acid sequence – as reported by Reich and Schoolnick – did not show significant homologies to other ADP–ribosylating enzymes. Nevertheless, a further investigation performed by our group put into evidence the presence of regions that align well with all the catalytic residues detected for the other toxins (Fig. 3).

E. Eukaryotic Mono-ADP–Ribosyltransferases

Eukaryotic mono-ADP–ribosyltransferases represent a growing class of enzymes. During the last decade, mono-ADP–ribosyltransferases from mammalian and avian cells have been cloned and characterized, and specific target proteins have been identified (Zolkiewska et al. 1994). Analysis of their deduced amino acid sequences have shown similarities to those of viral and bacterial toxin enzymes in the region of the active-site cleft, which is consistent with a common mechanism of NAD binding and ADP–ribose transfer (Domenighini and Rappuoli 1996). Recent studies suggest that, in vertebrates, this post-translational protein modification event may be used to control important endogenous physiological functions, such as the induction of long-term potentiation in the brain, terminal muscle cell differentiation and the cytotoxic activity of killer T cells (McMahon et al. 1993; Schuman et al. 1994; Wang et al. 1994).

The first vertebrate ribosyltransferases were purified and sequenced from chicken bone marrow and from rabbit and human skeletal muscle, and their specific target proteins have been identified. The majority of eukaryotic enzymes are arginine-specific transferases; nonetheless, ADP–ribosylation of cysteines was reported in bovine and human erythrocytes and platelet membranes (Saxty and Van Heyningen 1995).

The family of mammalian enzymes consists of five proteins (ART1–5), sharing extensive similarities in their gene structures and amino acid sequences (Okazaki and Moss 1998). The rabbit ART1 is a 36 kDa protein, and its deduced amino acid sequence possesses hydrophobic amino- and carboxy-terminal signal peptides that are characteristic of glycosylphosphatydylinositol (GPI)-linked proteins. There is roughly 75% sequence identity among ART1 muscle enzymes isolated from humans, rats and rabbits; this feature is consistent with considerable conservation across species. Like CT and LT, the muscle transferases specifically use the guanidino group of argi-

nine as an ADP–ribose acceptor. The α-7 integrin has been recently shown to be the target protein for cell-surface mono-ADP–ribosylation in muscle cells (ZOLKIEWSKA and Moss 1993).

The ART1 enzymes have significant amino acid sequence identity to the RT6 (ART2) family of rodent T cell differentiation and activation antigens (TAKADA et al. 1995). The expression of RT6 proteins appears in post-thymic lymphocytes and is restricted to peripheral T cells and intestinal intra-epithelial lymphocytes. In the mouse, there are two functional copies of the RT6 gene (RT6-1 and RT6-2; KOCHNOLITE et al. 1996; Fig. 3) located on chromosome 7. Rat and mouse ART2 sequences are roughly 80% identical while, in humans and chimpanzees, the ART2 genes contain three premature stop codons and, thus, appear not to be expressed. In humans, the role of ART2 may be assumed by other related ADP–ribosyltransferases.

ART3 and ART4 were recently cloned from human testis and spleen, respectively, and they contain several regions of sequence similarity with ART1. Moreover, the hydropathy profiles of the amino- and carboxyl-terminal sequences of ART3 and ART4 demonstrate hydrophobic signal sequences consistent with the possibility that these enzymes, like ART1, may be GPI-linked.

An ART5 complementary DNA was cloned from Yac-1 murine lymphoma cells, and its deduced amino acid sequence has similarities to those of other ART proteins in regions believed to be involved in catalytic activity (OKAZAKI et al. 1996b; Fig. 3). Soon after, the cloning and characterization of a new ADP–ribosyltransferase (Yac-2), again from lymphoma cells, was reported (OKAZAKI et al. 1996a); the nucleotide and deduced amino acid sequences of Yac-1 and Yac-2 enzymes are 58% and 33% identical, respectively. The Yac-2 protein is membrane bound but, unlike the Yac-1, appears not to be GPI anchored. Also in this case, the amino acid sequence of the Yac-2 transferase contains consensus regions common to several bacterial toxins and mammalian transferases and NAD glycohydrolases (Fig. 3).

Other mammalian ADP–ribosyltransferases have been purified from rat brain and adrenal medulla (FUJITA et al. 1995). These enzymes have been shown to modify, to different degrees, β/γ-actin, smooth-muscle γ-actin, G_s, G_i and G_0. The in vitro modification of brain and adrenal G proteins suggests potential mechanisms for cell signaling similar to those observed with the bacterial toxins.

Recent publications describe a novel enzymatic mono-ADP–ribosyltransfer reaction induced by a fungal metabolite, brefeldin A (BFA; DI GIROLAMO et al. 1995). This is a well-characterized protein that exerts generally inhibitory actions on membrane transport and causes the block of vesicular traffic in eukaryotic cells (COLANZI et al. 1997). In this case, the data obtained suggest a possible role for ADP–ribosylation in the Golgi-disassembling activity of BFA and also that the ADP–ribosylated substrates are components of the machinery controlling the structure of the Golgi apparatus (MIRONOV et al. 1997).

Although no significant overall sequence similarity could be detected between bacterial and eukaryotic mono-ADP ribosyltransferases, a significant homology in terms of primary sequence and predicted structural organization has nevertheless been shown in the region of the active-site cleft, with all the catalytic residues well conserved within the two groups (Fig. 3). This finding is consistent with a common mechanism of NAD binding and ADP–ribose transfer.

In computer-modeling studies of mouse ART2, it has been shown that Arg126 is localized on a β-strand (region 1), Ser147 on a β-strand followed by an α-helix (region 2) and the active site Glu184 on another β-strand (region 3), and that they are positioned in the catalytic cleft in a manner similar to that found in the crystal structure of the bacterial CT, LT and PT. In the alignment of deduced amino acid sequences of ART1, ART4 and ART5, a region-1 arginine and region-2 serine similar to those in LT and PT appear to be conserved. Based on site-directed mutagenesis and amino acid sequence alignment, the region containing Glu–X–Glu, present in ART1 and ART5, is postulated to be analogous to the region containing Glu110 and Glu112 of LT and CT.

Moreover, the deduced amino acid sequences of human PARPs (and perhaps ART3) appear to have regions of similarity that align with DT and PAETA. To support this observation, the crystal structure of chicken PARP (Ruf et al. 1996) and mutagenesis of human PARP (Marsischky et al. 1995) demonstrated that Glu988, which is essential for ADP–ribose-chain elongation, is positioned in a cleft similar to that found in bacterial toxins. These data are consistent with the hypothesis that the bacterial toxins and vertebrate transferases possess a common mechanism of NAD-binding and ADP–ribose transfer and that differences observed in the three-dimensional structures may reflect differences in substrate proteins.

F. Practical Applications

Computer analysis of the amino acid sequences of bacterial ADP–ribosylating toxins, supported by knowledge of the three-dimensional structures now available for some of them, demonstrated a striking level of conservation among all the catalytic domains. Table 1 and Fig. 3 report a comprehensive list of all the ADP–ribosyltransferases known and the alignment of their consensus sequences. The definition of this common "signature" led to a series of scientific discoveries and to important practical applications.

In the cases of LT and CT, computer modeling was used as a predictive tool in order to construct non-toxic derivatives of these molecules that could still be active as mucosal adjuvants and immunogens.

Before the structure was available, site-directed mutagenesis and biochemical studies had already identified a number of amino acids essential for enzymatic activity that are located within the catalytic cleft (Arg7, His44,

Ser61, Glu110 and Glu112). The availability of the three-dimensional structure of LT allowed researchers to study the A subunit in more detail and to identify additional amino acids that may have roles in catalysis.

Two of the more interesting mutants produced are LT-Ser63Lys (LTK63) and LT-Val97Lys (LTK97), for which X-ray structures have also been determined (Merritt et al. 1995; Van der Akker et al. 1997). In particular, LTK63 and the corresponding mutant obtained for CT (CTK63) show no detectable enzymatic activity and no toxicity in vitro or in vivo, even when huge amounts are used (Giannelli et al. 1997; Giuliani et al. 1998). X-ray structures, in fact, have shown complete identity to the wild type LT across the entire molecule, with the exception of the active site, where the bulky side chain of Lys63 fills the catalytic cavity, thus making it unsuitable for NAD entrance (Fig. 4A). Nonetheless, many other biological properties, including receptor and ADP–ribosylation-factor binding, are maintained intact (Stevens et al. 1999).

LTK63 is an excellent mucosal adjuvant, though the activity is reproducibly reduced in comparison with LT (Douce et al. 1997; Giuliani et al. 1998), while CTK63 is a less active mucosal adjuvant (Douce et al. 1997). In the case of LTK97, the Val→Lys substitution does not change the three-dimensional structure of the molecule but introduces a salt bridge between the charged amino group of Lys97 and the carboxylate of Glu112, thus making it unavailable for further interactions (Fig. 4B). The observation that a simple hydrogen bond inactivates the enzymatic activity suggests an important role in the enzymatic activity for the negative charge of the glutamic acid. In the case of PT, the roles of many amino acids of the S1 subunit have been tested using site-directed mutagenesis in order to produce non-toxic mutants to be used as vaccine candidates. Within the minimal region still enzymatically

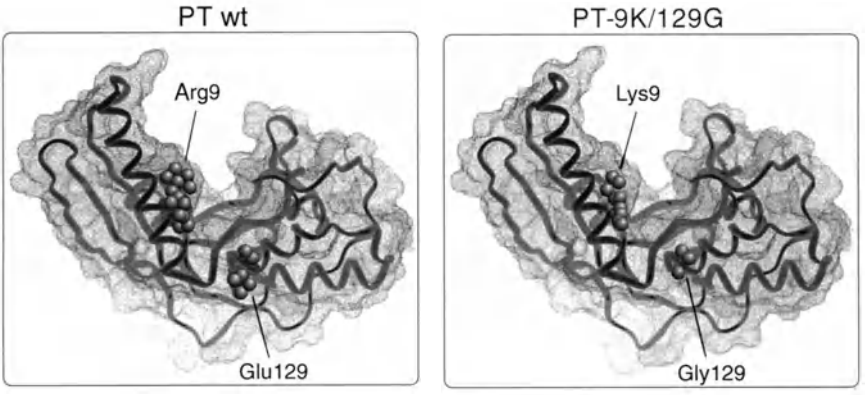


Fig. 5. Three-dimensional structure of the S1 subunit of the wild type pertussis toxin (PTwt *left panel*) and of the double mutant PT-9K/129G (*right panel*). *Arrows* indicate the catalytic residues (Arg9 and Glu129 in wild type PT) and the corresponding mutations (Lys9 and Gly129 in PT-9K/129G). The scaffolding structure of the enzymatic cleft is represented by the ribbons.

active, some amino acids have been shown to be essential; replacement of Arg9, Asp11, Arg13, Trp26, His35, Phe50, Glu129 or Tyr130, in fact, reduced the activity of recombinant S1 molecules to levels equal or below 1%.

The most interesting mutant contains two amino acids substitutions inside the catalytic cavity: Arg9Lys and Glu129Gly (PT9K/129G; Pizza et al. 1989; Fig. 5). This double mutant is completely free of any toxicity and has been used for the construction of an acellular vaccine against pertussis, which has been extensively tested in clinical trials and shown to induce protection from disease. The vaccine containing this mutant is presently licensed in several countries (Pizza et al. 1989; Rappuoli 1997).

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CHAPTER 3

Diphtheria Toxin and the Diphtheria-Toxin Receptor

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A. Introduction

The discovery of diphtheria toxin from culture medium of *Corynebacterium diphtheriae* was one of the greatest discoveries in medical history. Since this epoch-making change in medicine, diphtheria toxin has been studied, first to prevent disease and later to understand the pathogenic mechanism at the molecular level. A number of important discoveries in the fields of bacterial pathogenesis and bacterial protein toxins have resulted from these studies, such as: (1) antibodies directed against the toxin can protect against the disease, (2) diphtheria toxin acts within cells by inactivating a target protein by adenosine diphosphate (ADP)–ribosylation, and (3) the toxin consists of two segments with distinct functions. Because of extensive studies over a long period, diphtheria toxin is one of the best known of the bacterial protein toxins, and discoveries, such as those enumerated above, have frequently led to increased understanding of the biology of other bacterial toxins.

To fully understand the intoxication process, it is necessary to know the host cell factors involved and to determine how they participate in the intoxication process. Although various cellular factors are involved in intoxication, a cell-surface receptor for diphtheria toxin is one of the key factors that determine the sensitivity of cells to this toxin. Until recently, less was known about diphtheria-toxin receptor, but research over the last several years has enabled study of the diphtheria-toxin receptor, and the interaction of diphtheria toxin with its receptor has recently been characterized at the molecular level. In addition, the physiological role of diphtheria-toxin receptor in cells is much more clearly understood due to recent studies. In this review, we have tried to emphasize the recent advances in the understanding of diphtheria toxin and diphtheria-toxin receptor.

B. Diphtheria Toxin

I. Synthesis of Diphtheria Toxin

Diphtheria toxin is synthesized in *C. diphtheriae* lysogenized by a bacteriophage carrying the *tox* structure gene (FREEMAN 1951; UCHIDA et al. 1971). Under optimal conditions for toxin production, diphtheria toxin is secreted

and accumulated by the PW8 strain in a culture medium; it reaches a final concentration of ~400–500 μg/ml, ~75–90% that of the extracellular proteins and to ~5% that of the bacterial proteins. It is well known that adding iron at low concentration to the growth medium inhibits the production of diphtheria toxin (Pappenheimer 1977). Diphtheria-toxin repressor gene (*dtxR*) is known to regulate transcription of the diphtheria-toxin gene (*tox*) by an iron-dependent mechanism (Murphy et al. 1976; Boyd et al. 1990). The *tox* operator locus was found to bear sticking homology to Fur (ferric-uptake regulator)-binding sites in the *Escherichia coli* chromosome (Calderwood and Mekalanos 1987; De Lorenzo et al. 1987; Tai and Holmes 1988).

II. Toxicity of Diphtheria Toxin

Diphtheria toxin is produced as a single polypeptide chain with a molecular weight (MW) of 58,348 Da, with two disulfide bridges (Collier and Kandel 1971; Gill and Dinius 1971). The native toxin is called intact toxin. The toxin molecule is easily hydrolyzed by splitting a peptide bond between one of the disulfide bridges by mild treatment with trypsin, trypsin-like proteases (Gill and Dinius 1971), or furin on the host cell membrane (Tsuneoka et al. 1993). This form of toxin is called nicked toxin. On reduction of its disulfide bridges with a reducing agent, nicked toxin is split into two large peptides, fragment A (MW = 21,167 Da) and fragment B (MW = 37,199 Da). The toxicity of diphtheria toxin is due to the enzymic activity of fragment A. Fragment A catalyzes the transfer of an ADP–ribosyl moiety from nicotinamide adenine dinucleotide (NAD) to a post-translationally modified histidine, diphthamide, on elongation factor 2 (EF-2; Collier 1967; Honjo et al. 1968). EF-2 is a GTP-binding protein involved in protein synthesis by eukaryotic cells. ADP–ribosylated EF-2 is unable to mediate polypeptide-chain elongation, resulting in inhibition of protein synthesis.

Since fragment A alone is unable to penetrate the cell membrane, it cannot reach the cytosol of intact cells without the involvement of fragment B. Fragment B binds to the specific receptor of toxin-susceptible cell membranes (Uchida et al. 1972) and facilitates translocation of fragment A into the cytosol. The first step of the intoxication is the binding of the toxin by fragment B to the receptor (Fig. 1). Thereafter, receptor-bound toxin is internalized by endocytosis. The interiors of endosomes are kept acidic by vacuolar-type H^+–adenosine triphosphate (ATP)ases. A conformational change of the toxin takes place in the acidic compartment, resulting in the exposure of hydrophobic domains (Sandvig and Olsnes 1980; Blewitt et al. 1985; Cabiaux et al. 1989), which are mainly in the fragment B (Boquet et al. 1976; Greenfield et al. 1983), and insertion of the hydrophobic domains to membranes (Silverman et al. 1994). Finally, the enzymatically active fragment A is translocated to the cytosol, where it exerts its toxicity (Moskaug et al. 1991; Umata and Mekada 1998).

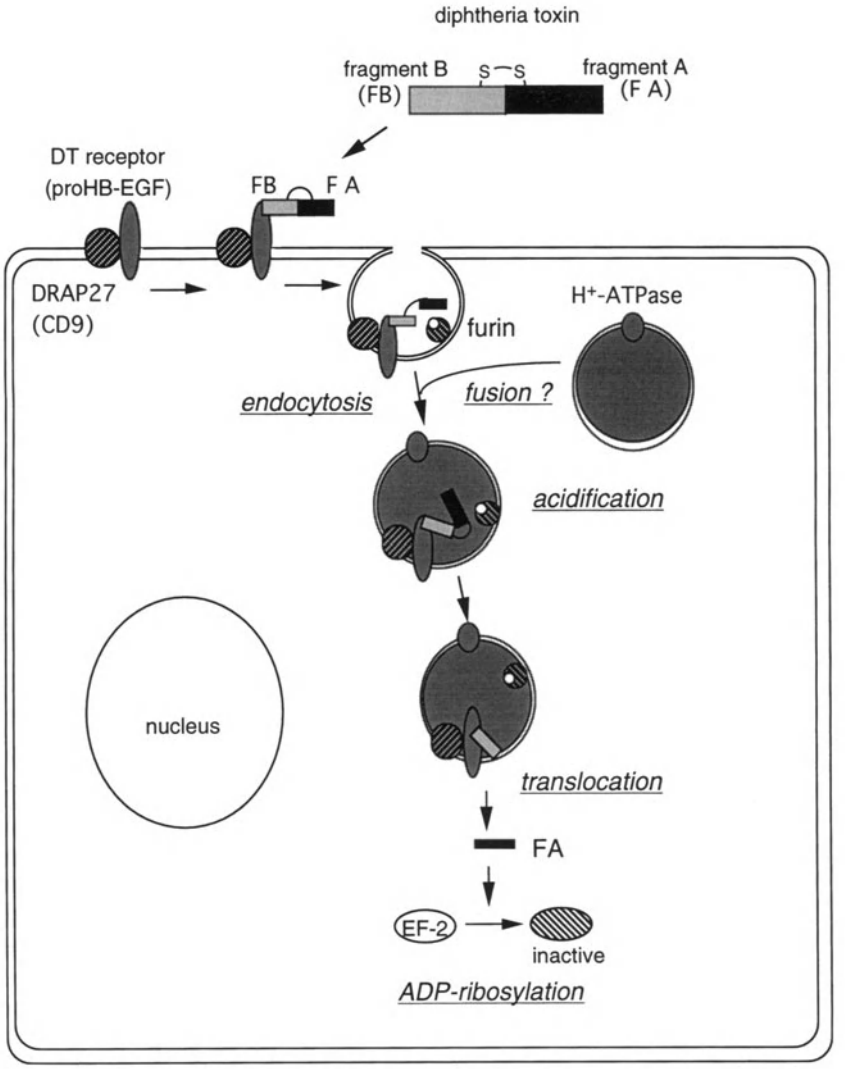

Fig. 1. Process of diphtheria-toxin intoxication

Fragment A is quite toxic once it reaches the cytoplasm. It is known that one molecule of fragment A can kill a cell when fragment A is artificially introduced directly into the cytosol (YAMAIZUMI et al. 1978). This remarkably strong toxicity of fragment A of diphtheria toxin could be explained by the following: (1) fragment A is quite stable and has a long half-life in the cytosol of cultured cells, (2) fragment A catalytically inactivates EF-2 in the cytosol, and (3) ADP–ribosylated EF-2 would have a dominant-negative effect on the native, functional EF-2.

III. Structure and Function of Diphtheria Toxin

The crystal structure of diphtheria toxin (Choe et al. 1992) reveals that diphtheria toxin is a Y-shaped molecule divided into three domains, i.e., the C (catalytic), T (transmembrane), and R (receptor-binding) domains, rather than two fragments.

1. The Catalytic Domain

The C domain (amino acids 1–192), or fragment A, forms both α-helices and β-sheets (Choe et al. 1992). Several lines of evidence indicate that glu148 of this domain is a crucial residue in the active site of the enzyme (Carroll and Collier 1984; Carroll et al. 1985). His21 and Tyr65, both of which are involved in NAD binding (Papini et al. 1989, 1991; Blanke et al. 1994), are also located at the active-site cleft. CRM197 is a non-toxic mutant of diphtheria toxin (Uchida et al. 1972) with a substitution of Gly52 with Glu (Giannini et al. 1984). Gly52 is also located near the active site. Recent studies of the crystal structures of other ADP–ribosylating toxins indicated that the enzymatically active domains of ADP–ribosylating toxins have a universal feature in their tertiary structures in spite of the low degree of homology at the amino acid level (Chap. 2).

2. The T Domain

The T domain (amino acids 193–381), a very strong hydrophobic domain corresponding to the N-terminal half of fragment B, is involved in the translocation of fragment A across the membrane. In an acidic environment, the T domain is inserted into membrane and forms an ion-conducting channel (Donovan et al. 1981; Kagan et al. 1981). This domain is formed by nine α-helices (TH1-TH9) arranged in three layers (Choe et al. 1992; Collier 1994). TH8, TH9, and the connecting loop (TL5) form the innermost layer. Two acidic residues, E349 and D352, located in the tip of TL5, have a critical role for low-pH-mediated insertion of the T domain into the membrane (O'Keefe and Collier 1989; Mindell et al. 1992; Silverman et al. 1994). Thus, substitution of Lys for Glu349 or Asp352 reduced translocation through plasma and endosome membranes, resulting in reduced toxicity. The second layer consists of three hydrophobic helices, TH5, TH6, and TH7. Although the role of these helices in the translocation of fragment A has been less characterized compared with that of TH8/9 helices, it is likely that they form a channel together with TH8/9 helices. The fragment A molecule passes through this channel when these helices are inserted into the membrane.

The third layer, consisting of three helices, TH1, TH2, and TH3, are located in the outermost position. These helices contain a number of charged residues and probably protect the other hydrophobic helices from aqueous environment to keep the diphtheria toxin molecule in a water-soluble form. Because of the hydrophilic nature of these helices, this domain is not likely to be

inserted into a lipid bilayer of the membrane by direct lipid–protein interaction. However, mutation studies of TH1 indicate the importance of these helices for the translocation of fragment A into the cytoplasm (MADSHUS et al. 1991; VANDERSPEK et al. 1993, 1994). These results imply that TH1 may not take part in the formation of translocation channel which TH8/9 and TH5/6/7 could form in phospholipid membrane; TH1 has another role during the translocation process of fragment A (VANDERSPEK et al. 1994).

Using a cell-permeabilization method, we recently developed a novel assay for the translocation of fragment A across the endosomal membrane into the cytosol (UMATA and MEKADA 1998). This assay revealed novel fragments of diphtheria toxin having masses of 28 kDa and 35 kDa in endocytic vesicles. The 28-kDa fragment is comprised of fragment A and an N-terminal piece of fragment B, while the 35-kDa fragment is another part of fragment B and might be the counterpart of the 28-kDa fragment. The length of N-terminal piece of fragment B in the 28-kDa fragment is about 3 kDa, estimated as ~30 amino acids, which would include the TH1 region of diphtheria toxin. Although it is possible that the 28-kDa and the 35-kDa fragments are products of a degradation process and that they failed to translocate to the cytoplasm, it is tempting to infer that the 28-kDa fragment is an intermediate during the translocation of fragment A. From what is known about the TH8/9 and TH1 helices, it is assumed that the T domain of diphtheria toxin consists of two functionally-separable domains; one serves as translocation machinery and the other serves as a translocation signal. TH8/9 (and probably TH5/6/7) would form an acid-induced channel in the endocytic membrane; this would serve as translocation machinery for fragment A. TH1 (either with or without TH2/3) serves as a kind of signal or recognition sequence for translocation of fragment A for interaction with translocation machinery, including TH8/9 and TH5/6/7, at the initial stage of translocation.

3. The R Domain

The R domain (amino acids 386–535), the domain corresponding C-terminal half of fragment B, possesses the ability to bind to the diphtheria-toxin receptor, is constructed of two β-sheets arranged in a quasi-jellyroll configuration, and resembles the immunoglobulin variable region (CHOE et al. 1992). Peptide fragments corresponding to the last 54 residues of the R domain (amino acids 482–535) competitively inhibit the binding of diphtheria toxin to Vero cells (ROLF et al. 1990). Analysis of mutant toxins unable to bind to cells showed that substitutions of S390F, S508F, and S525F are observed (GREENFIELD et al. 1987). Systematic replacement with alanine residues within C-terminal region of diphtheria toxin (residue 516–530) indicated that Lys516 and Phe530 are important for receptor binding (SHEN et al. 1994). These results suggested that receptor-binding site of diphtheria toxin lies within the C-terminal 54 residues of R domain.

More direct information has been obtained by analysis of the crystal structure of the complex of diphtheria toxin and an extracellular fragment of the diphtheria-toxin receptor (Louie et al. 1997). The structure of the diphtheria toxin–diphtheria-toxin-receptor complex revealed that the crescent-shaped diphtheria-toxin-receptor molecule is packed against a saddle-shaped crevice in the wall of the β-barrel of the R domain of diphtheria toxin. The complex forms an intermolecular interface with predominantly non-polar amino acids. Non-polar surfaces are formed on the diphtheria toxin by the side chains of F389, A430, L433, I464, V468, F470, G510, L512, V523, and F530. K526, an exception to the non-polarity rule, is also located in the core of the interface. H391 and K516 form important hydrogen bonds with E141 of the diphtheria-toxin receptor, which is a critical residue for the diphtheria-toxin-binding activity of this molecule, as mentioned in later sections (Mitamura et al. 1995, 1997). F530, which interacts with G137 of the diphtheria-toxin receptor, is important in closing off one end of the interface between the two molecules, which is consistent with earlier observations of diphtheria-toxin deletion mutants.

IV. Sensitivity to Diphtheria Toxin

Diphtheria toxin is highly toxic to most animals, including rabbits, guinea pigs, monkeys, and humans. The lethal dose for susceptible animals is 100 ng/kg body weight or less (Collier 197; Pappenheimer 1977). The only mammals known to be resistant to the toxin are rats and mice. These animals are about 1000 times more resistant than susceptible species. Generally speaking, cells derived from sensitive species are sensitive to the cytotoxic action of diphtheria toxin, while cells from toxin-resistant species are quite resistant to its action (Lennox and Kaplan 1957; Placido-Sousa and Evans 1957; Gabliks and Solotorovsky 1962; Middlebrook and Dorland 1977). There are quite large differences in sensitivity between diphtheria-toxin-sensitive cells and resistant cells. For example, Vero cells derived from monkey kidney are about 100,000 times more sensitive than mouse L cells (Mekada et al. 1982). Among cells derived from susceptible species, the sensitivity to diphtheria toxin varies from cell line to cell line, as shown in Fig. 2, and some human cell lines are known to be quite resistant to diphtheria toxin.

So far, EF-2 from any eukaryote is inactivated in vitro by fragment A of diphtheria toxin. Thus, the resistance of cells to diphtheria toxin is regarded to be generally due to the binding and entry process. Indeed, in the case of diphtheria-toxin-resistant L cells, one molecule of fragment A introduced into the cytoplasm can kill a cell, as mentioned before (Yamaizumi et al. 1978). A number of cellular factors are involved in the intoxication process and could influence toxin sensitivity. One example is furin, a membrane-bound serine protease (Tsuneoka et al. 1993). Furin serves to cleave diphtheria toxin into fragment A and fragment B during and after binding of intact toxin to the receptor. Intact toxin has no catalytic activity in ADP-ribosylation and may also fail to translocate into the cytosol. Thus, intact toxin shows quite low toxi-

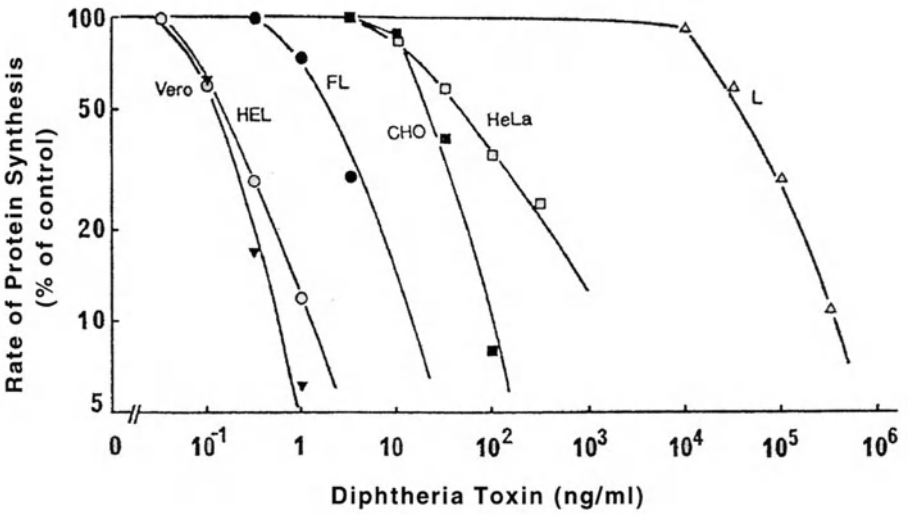

Fig. 2. Sensitivity of various cell lines to diphtheria toxin, assayed by measuring the inhibition of protein synthesis (Mekada et al. 1982)

city in furin-deficient cells, while both forms of toxin normally show similar toxicities. The other example is a vacuolar-type H^+–ATPase. A vacuolar-type H^+–ATPase has a crucial role for entry of this toxin (Umata et al. 1990). A vacuolar-type H^+–ATPase is the primary molecule for acidification of intracellular compartments. Diphtheria toxin requires exposure to an acidic milieu for translocation of fragment A into the cytosol. Therefore, bafilomycin A1, a specific inhibitor of vacuolar-type H^+–ATPase, and amine compounds that accumulate into the acidic compartments elevate their pH and inhibit the toxicity of diphtheria toxin (Kim and Groman 1965; Draper and Simon 1980; Sandvig and Olsnes 1980; Mekada et al. 1981). Most of these factors, however, are essential for normal cellular function and are inherent even in mouse and rat cells; thus, these factors are unlikely to be the determining factor for diphtheria-toxin sensitivity, except in some mutant cell lines. One exception is diphtheria-toxin receptor; cells from rats and mice have no functional diphtheria-toxin receptor that specifically binds to diphtheria toxin, as discussed below. Thus, in many types of cells, cell sensitivity to diphtheria toxin is primarily determined by the presence of functional diphtheria-toxin receptors.

C. The Diphtheria-Toxin Receptor

I. Identification of the Diphtheria-Toxin-Receptor Protein

The existence of a specific receptor for diphtheria toxin on the cell surface was suggested by competition experiments using CRM197, a non-toxic mutant of diphtheria toxin (Itelson and Gill 1973). Then, using ^{125}I-labeled diphtheria

toxin and Vero cells, specific binding of diphtheria toxin to cultured cells was demonstrated (MIDDLEBROOK et al. 1978). However, it took about 10 years to identify the diphtheria-toxin receptor in detergent-treated cell fractions at a protein level (MEKADA et al. 1988).

The reason for the difficulty in identifying and purifying the specific receptor for diphtheria toxin from cell fractions was the existence of another diphtheria-toxin-binding substance in membrane fractions (MEKADA et al. 1988). This non-receptor diphtheria-toxin-binding molecule contains a ribonucleotide structure. Diphtheria toxin tightly binds some dinucleotides, such as adenyl-(3′,5′)-uridine-3′-monophosphate (COLLINS and COLLIER 1984). ATP also binds to diphtheria toxin (LORY and COLLIER 1980) and inhibits its cytotoxic activity (MIDDLEBROOK and DORLAND 1979). These non-receptor diphtheria-toxin-binding substances inhibit the binding of toxin to the specific receptor. CRM197, a mutant protein of diphtheria toxin, differs from wild-type toxin in one amino acid residue of fragment A (GIANNINI et al. 1984). CRM197 does not bind nucleotides or the non-receptor diphtheria-toxin-binding substances (MEKADA et al. 1988) but binds to diphtheria-toxin receptor with an affinity similar to or greater than that of diphtheria toxin (MEKADA and UCHIDA 1985).

We demonstrated specific binding of CRM197 to the diphtheria-toxin receptor in a membrane preparation from Vero cells (MEKADA et al. 1988). Scatchard analysis showed single-class binding with a K_d value of $2.4 \times 10^9 M^{-1}$, quite similar to that obtained with intact Vero cells. Using a combination of several chromatographic steps, diphtheria-toxin receptor has been purified in the presence of detergent (MEKADA et al. 1991). The purified receptor essentially showed a single band of 14.5 kDa by sodium dodecyl sulfate polacrylamide-gel electrophoresis. Evidence that the 14.5-kDa protein is the receptor, or at least a part of the receptor, was obtained by immunoprecipitation with diphtheria toxin and anti-diphtheria-toxin antibody from the partially purified receptor fraction or by blotting using ^{125}I–CRM197 as a probe (MEKADA et al. 1988). Although the 14.5-kDa protein was the major form isolated from Vero cell-membrane lysate, other sizes of diphtheria-toxin-binding molecules (~20 kDa and ~17 kDa) were detected in different fractions from CM–Sepharose ion-exchange chromatography (MEKADA et al. 1991). The 14.5-kDa protein and the 17-kDa and 20-kDa proteins are derived from a single precursor protein, as discussed below.

II. Cloning of the Diphtheria-Toxin-Receptor Gene

Complementary DNA (cDNA) encoding the diphtheria-toxin receptor has been isolated as a diphtheria-toxin-sensitivity gene by expression cloning (NAGLICH et al. 1992). Mouse L cells were transfected with a cDNA library obtained from monkey kidney (Vero) cells. Transfectants that were sensitive to diphtheria toxin were isolated using a replica plate assay (NAGLICH and EIDELS 1990). A cDNA (pDTS) was recovered from the diphtheria-toxin-

sensitive transfectants. Mouse L cells transfected with this cDNA become sensitive to diphtheria toxin and display diphtheria toxin-binding molecules on their cell surface that have the characteristics of the diphtheria-toxin receptor (NAGLICH et al. 1992). The predicted protein product of pDTS has 185 amino acids, a quite basic isoelectric point, and characteristics of an integral membrane protein. These characteristics are consistent with the characteristics of the diphtheria-toxin-receptor protein isolated by us (MEKADA et al. 1991).

Homology-search analysis showed that the product of pDTS is identical to heparin-binding epidermal growth factor, HB-EGF (NAGLICH et al. 1992), first identified by HIGASHIYAMA et al. (1991). Further evidence that HB-EGF is the diphtheria-toxin receptor has been obtained from direct-binding experiments with diphtheria toxin and a recombinant human HB-EGF (IWAMOTO et al. 1994). A mature form of HB-EGF, produced in *E. coli*, was immobilized on heparin–Sepharose beads, and binding of diphtheria toxin to the recombinant HB-EGF was studied. The amount of ^{125}I–diphtheria toxin bound was a linear function of the amount of immobilized recombinant HB-EGF, and saturation of binding occurred when a fixed amount of immobilized HB-EGF was incubated with increasing amounts of diphtheria toxin. Scatchard analysis demonstrated that diphtheria toxin binds to HB-EGF with an affinity similar to that for intact Vero cells (K_a of about $1+10^9\,M^{-1}$). These results clearly show that the diphtheria-toxin receptor is HB-EGF.

III. The Structure and Function of the Diphtheria-Toxin Receptor

Diphtheria-toxin receptor/HB-EGF cDNA encodes a protein of 208 amino acids (HIGASHIYAMA et al. 1991; NAGLICH et al. 1992). This protein consists of a characteristic signal sequence of 23 amino acid residues, a presumed extracellular domain of 136 residues (24–159), a putative transmembrane domain of 25 residues (160–184), and a C-terminal cytoplasmic domain of 24 residues (185–208; Fig. 3). The mature protein, after cleavage of the signal peptide, seems to be comprised of 185 amino acids with a calculated molecular weight of 20,652 Da. The extracellular domain includes two characteristic features: (1) an EGF-like domain with six cysteine residues with highly conserved spacing and (2) a heparin-binding domain with a highly basic stretch of amino acid residues upstream of the EGF-like domain.

Because HB-EGF is expressed with a very similar tissue distribution in multiple tissues in rats, mice, and humans (ABRAHAM et al. 1993), the question of why cells from rats and mice are resistant to diphtheria toxin arises. Transfection of human diphtheria-toxin receptor/HB-EGF cDNA into mouse L cells confers sensitivity to diphtheria toxin (NAGLICH et al. 1992), but transfection of mouse HB-EGF cDNA does not (MITAMURA et al. 1995). These results indicate that mouse HB-EGF does not serve as a functional receptor for diphtheria toxin because of amino acid substitution. To define the essential regions of HB-EGF that serve as the functional diphtheria-toxin receptor, MITAMURA et al. (1995) constructed a series of human/mouse

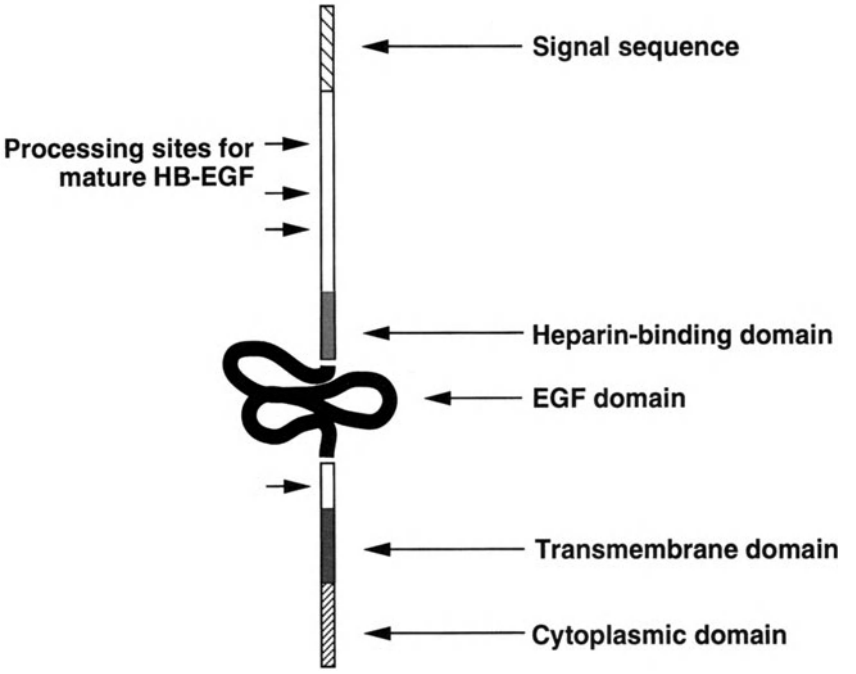

Fig. 3. Schematic structure of the diphtheria-toxin receptor

HB-EGF chimeras, and both sensitivity to diphtheria toxin and the diphtheria-toxin binding of cells expressing these chimeras were examined. These results clearly indicated that the EGF-like domain of human HB-EGF is essential for diphtheria-toxin binding and diphtheria-toxin sensitivity. However, mouse HB-EGF does not serve as a functional diphtheria-toxin receptor, due to non-conserved amino acid substitutions in this domain.

Diphtheria-toxin receptor, i.e., the membrane-anchored form of HB-EGF (proHB-EGF), is cleaved upstream of the transmembrane domain by a cell-associated protease (discussed more in a later section), and the extracellular domain is released into the medium as a soluble growth factor (Higashiyama et al. 1991, 1992). The secreted HB-EGF protein (sHB-EGF) is also processed at multiple sites in the N-terminal portion (Nakagawa et al. 1996). Thus, multiple forms of HB-EGF with different molecular sizes are secreted. HB-EGF secreted into culture media binds to diphtheria toxin (Iwamoto et al. 1994). Thus, the diphtheria-toxin-binding site lies wholly or partially within this region. The heparin-binding domain, which is located just upstream of the EGF-like domain, is not necessary for binding to diphtheria toxin, because deletion mutants lacking this domain bind diphtheria toxin with affinities similar or higher than that of wild type HB-EGF (Mitamura et al. 1995). These results, together with results of human/mouse HB-EGF chimeras, indicate that the EGF-like domain is essential and enough to bind diphtheria toxin.

Results of human/mouse HB-EGF chimeras provide information about important amino acids for diphtheria-toxin–diphtheria-toxin-receptor interaction. There are ten amino acid differences between the EGF-like domain of human diphtheria-toxin receptor/HB-EGF and that of mouse HB-EGF. We made ten independent mutants, replacing a single amino acid within the EGF-like domain of human diphtheria-toxin receptor/proHB-EGF with the corresponding amino acid residue in mouse proHB-EGF (Mitamura et al. 1997). The mutant proteins were transiently expressed in mouse L cells, and the diphtheria-toxin binding was measured. Glutathione *S*-transferase fusion proteins containing the mutated EGF-like domain were also examined. The largest effect was observed with E141H, and the second largest effects were with F115Y and L127F in all of the assay systems. Few but significant effects were also observed with I133K and H135L. A computer model of the tertiary structure of the EGF-like domain of human diphtheria-toxin receptor/proHB-EGF, based on the tertiary structure of transforming growth factor α (TGFα), was made. The model predicts that three amino acid residues critical for diphtheria-toxin-binding activity, Phe115, Leu127, and Glu141, are all located on the same face of the EGF-like domain, suggesting that this face of diphtheria-toxin receptor/proHB-EGF interacts with the receptor-binding domain of diphtheria toxin. This finding is supported by another report (Hooper and Eidels 1996). The crystal structure of diphtheria toxin-HB-EGF (Louie et al. 1997), which is quite consistent with earlier observations, has defined the complex structure more precisely.

IV. Molecules Associated with the Diphtheria-Toxin Receptor

1. DRAP27/CD9

Iwamoto et al. (1991) isolated a monoclonal antibody (mAb007) that inhibits the binding of diphtheria toxin to intact Vero cells. This antibody does not inhibit the binding of diphtheria toxin to solubilized receptors, suggesting that membrane integrity is necessary for the inhibition. Immunoprecipitation and Western-blot analysis revealed that this antibody recognizes a membrane protein with a mass of 27 kDa (DRAP27) and does not bind to diphtheria-toxin receptor. Immunoprecipitation studies of cell lysates prepared from cells overexpressing diphtheria-toxin receptor with either anti-DRAP27 antibody or CRM197 revealed that diphtheria-toxin receptor forms a complex with DRAP27 (Iwamoto et al. 1994). mAb007 Probably binds to DRAP27 molecules closely associated with diphtheria-toxin receptor on the cell surface and causes inhibition of the binding of diphtheria toxin to the receptor. Analysis of the nucleotide sequence of a DRAP27 cDNA has shown that DRAP27 has 228 amino acids, contains four putative transmembrane domains, and is the monkey homologue of the human CD9 antigen (Mitamura et al. 1992).

The role of DRAP27 on diphtheria-toxin binding and diphtheria-toxin sensitivity has been studied by transfection of DRAP27 cDNA to cells. A

human–mouse hybrid cell line (3279-10) expressing diphtheria-toxin receptor (Hayes et al. 1987) but not DRAP27/CD9 antigen was transfected with cDNA for DRAP27. The transfectants showed increased diphtheria-toxin binding and were 3–25 times more sensitive to diphtheria toxin than non-transfected cells (Mitamura et al. 1992). However, when mouse L cells with no functional diphtheria-toxin receptor were transfected with DRAP27 cDNA, neither increased diphtheria-toxin binding nor enhancement of diphtheria-toxin sensitivity was observed. Thus, DRAP27 serves to enhance the sensitivity of diphtheria-toxin-sensitive cell lines.

Co-transfection of DRAP27 cDNA and diphtheria-toxin-receptor cDNA into mouse L cells showed more clearly the role of DRAP27 (Iwamoto et al. 1994). L cells transfected transiently with both DRAP27 and diphtheria-toxin-receptor cDNA bound about 10 times more diphtheria toxin than cells transfected with diphtheria-toxin receptor alone. Transcription of diphtheria-toxin-receptor messenger RNA (mRNA) was not increased by co-transfection of DRAP27 cDNA. Stable L cell transfectants expressing both diphtheria-toxin receptor and DRAP27 had a 15-fold greater cell-surface diphtheria-toxin-receptor number and were 20 times more sensitive to diphtheria toxin than stable L-cell transfectants expressing diphtheria-toxin receptor alone, though the cell lines contained similar levels of diphtheria-toxin-receptor mRNA. Thus, DRAP27 upregulates diphtheria-toxin-receptor number and diphtheria-toxin sensitivity through protein–protein interaction, though the upregulation mechanism is still not clear (Fig. 4).

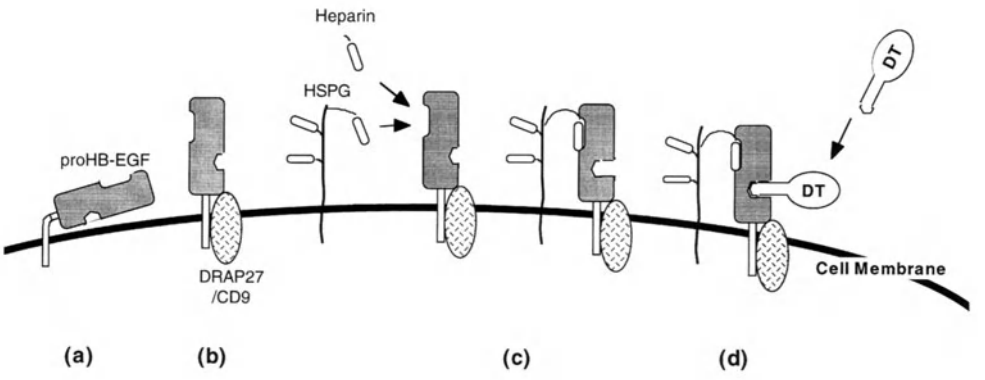

Fig. 4. Proposed model for the roles of DRAP27/CD9 and heparin-like molecules in binding of diphtheria toxin (DT) to DT receptor, the membrane-anchored form of heparin-binding epidermal growth factor (DTR/proHB-EGF). **a** DTR/proHB-EGF alone in the plasma membranes is not available for DT binding. **b** DRAP27/CD9 binds and orients DTR/proHB-EGF, making it accessible to DT. **c** Cell-surface heparan-sulfate proteoglycans (HSPGs) or free heparin bind to DTR/proHB-EGF at the heparin-binding domain and induce a conformational change which results in increased affinity of the DTR/proHB-EGF molecule for DT. **d** The DTR/proHB-EGF–DRAP27/CD9–HSPG–DT complex (Shishido et al. 1995)

2. Heparin-Like Molecules

Diphtheria-toxin receptor/proHB-EGF has a high affinity for heparin. The following evidence indicates that heparin-like molecules bind to the heparin-binding domain (HBD) of diphtheria-toxin receptor/proHB-EGF and enhance its diphtheria-toxin-binding activity. Mutant Chinese hamster ovary (CHO) cells deficient in heparan-sulfate proteoglycans were about 15 times less sensitive to diphtheria toxin than wild-type CHO-K1 cells. When free heparan sulfate or heparin was added to the culture medium, the diphtheria-toxin sensitivity of the mutant cells was fully restored (Shishido et al. 1995). Studies of the binding of ^{125}I-labeled diphtheria toxin to heparan-sulfate-deficient CHO cells transfected with human diphtheria-toxin receptor/proHB-EGF cDNA indicated that the increased sensitivity to diphtheria toxin after addition of heparin is due to increased binding of diphtheria toxin to cells. Vero cells display a relatively large amount of heparan-sulfate residues compared with CHO-K1 cells or L cells. Enhancement of diphtheria-toxin binding by the addition of heparin was also observed with CHO-K1 cells and L cells that had been transfected with human diphtheria-toxin receptor/proHB-EGF cDNA, but the degree of enhancement was less than that observed with the heparan-sulfate-deficient CHO cells. Scatchard plot analysis for the binding of diphtheria toxin to a recombinant HB-EGF in vitro and to L cells expressing human diphtheria-toxin receptor/proHB-EGF revealed that heparin increases the affinity of diphtheria-toxin receptor/proHB-EGF for diphtheria toxin but does not change the number of binding sites. Although DRAP27/CD9 is known to enhance diphtheria-toxin binding to diphtheria-toxin receptor/proHB-EGF, the results indicate that heparin and DRAP27/CD9 increase diphtheria-toxin binding by independent mechanisms. Addition of heparin did not affect diphtheria-toxin binding or diphtheria-toxin sensitivity of Vero cells, probably due to the relative abundance of heparan-sulfate molecules on the cell surface. In fact, heparin-dependent binding was observed when intact Vero cells were treated with heparitinase or when the cell membrane was solubilized with a neutral detergent. Thus, it is conceivable that diphtheria-toxin receptor/proHB-EGF associates with heparan-sulfate proteoglycans at the HBD. The diphtheria-toxin-binding activity of the receptor was enhanced when this protein associates with heparin-like molecules.

Diphtheria-toxin receptor binds diphtheria toxin at the EGF-like domain, and this domain is essential and sufficient for the binding of diphtheria toxin, as mentioned in Sect. III, while binding of heparin-like molecules to HBD is necessary for the full binding activity of diphtheria-toxin receptor with diphtheria toxin when assayed in intact cells. To explain this discrepancy, heparan-sulfate-deficient CHO cells were transfected with proHB-EGF mutants lacking HBD, and the effect of heparin or heparan sulfate on the binding of diphtheria toxin was examined. In the absence of heparin-like molecules, proHB-EGF mutants lacking HBD have a higher affinity for diphtheria toxin than wild-type proHB-EGF does. By addition of heparin or

heparan sulfate, diphtheria-toxin binding was much increased in wild-type proHB-EGF but didn't increase much in mutant proHB-EGF lacking HBD (MEKADA, unpublished observation). These results suggested that HBD is necessary for binding to diphtheria toxin itself, and plays the role of a negative regulator for the binding of diphtheria toxin to the EGF-like domain of proHB-EGF. Heparin and heparan-sulfate proteoglycans cancel or neutralize the negative effect of HBD by binding to the HBD domain.

V. Receptor and Toxin Entry Process

The way in which fragment A of diphtheria toxin penetrates the endosome membrane and reaches the cytosol is an important and intriguing issue in the understanding of intoxication mechanisms of many protein toxins and diphtheria toxin. Since more expert reviews about uptake of bacterial protein toxins have been shown in Chap. 1, we mention here the issue of diphtheria-toxin entry from the standpoint of diphtheria-toxin–diphtheria-toxin-receptor interaction.

Diphtheria-toxin molecules bound to the cell-surface receptor are internalized by receptor-mediated endocytosis (MORRIS et al. 1985; MOYA et al. 1985). Diphtheria-toxin receptor, in particular its C-terminal region (located on the cytoplasmic side), would be expected to exhibit a signal for internalization or a site that interacts with endocytosis machinery. Tyrosine residues in the cytoplasmic domain have been shown to be important for rapid endocytosis in other receptors (DAVIS et al. 1987). Diphtheria-toxin receptor has two tyrosine residues in the cytoplasmic domain, and it has been suggested that one of these, Tyr192, is in a region that resembles the signal necessary for receptor-mediated endocytosis (NAGLICH et al. 1992). Analysis of cells expressing diphtheria-toxin-receptor mutants with altered cytoplasmic domains indicated that the internalization signal might be present in another region or that it does not exist, particularly in the diphtheria-toxin receptor (ALMOND and EIDELS 1994). It is also conceivable that internalization of diphtheria-toxin receptor is accompanied by diphtheria-toxin receptor-associating molecules that possess the internalization signal.

In the endocytic vesicles, diphtheria toxin still seems to be bound to the receptor. Binding assays of diphtheria toxin with solubilized diphtheria-toxin receptor in cell-free conditions showed that solubilized diphtheria-toxin receptor is not eluted from a diphtheria-toxin-conjugated column by the addition of solutions at pHs below 3.5 (MEKADA, unpublished observation). The pH of the endosome interior is around 5.0, so diphtheria toxin could remain bound to the receptor in the endosomal compartment. In the acidic environment of the endosome, the T domain of diphtheria toxin, adjacent to the R domain, is inserted into the endosome membrane to translocate the fragment A. If the R domain remains bound to the receptor molecule, even at this stage, the T domain of the toxin may be close to the transmembrane domain of the diphtheria-toxin receptor in the endosome bilayer. It is possible that the

diphtheria-toxin receptor affects the translocation step of diphtheria toxin. A more interesting possibility is that DRAP27/CD9 or other TM4SF proteins are involved in the translocation step, because these proteins are integral membrane proteins with four transmembrane domains, and they look like channel-forming proteins. Whether diphtheria toxin utilizes diphtheria-toxin receptor and/or other associating proteins when the toxin penetrates the endosome membrane is an intriguing question.

VI. Physiological Role of the Diphtheria-Toxin Receptor

1. EGF-Family Growth Factor

HB-EGF was first identified and purified as a soluble growth factor in a conditioned medium of the human monocyte/macrophage-like cell line U-937 (Higashiyama et al. 1991, 1992). Secreted mature HB-EGF polypeptide contains at least 86 amino acid residues, while the precursor molecule has 208 amino acids, which includes a putative N-terminal signal sequence and a hydrophobic T domain near the C-terminus, as described in Sect. III. HB-EGF belongs to the EGF family of growth factors (Carpenter and Wahl 1990), which includes EGF, TGFα (Lee et al. 1985), amphiregulin (Shoyab et al. 1989), and β-cellulin (Shing et al. 1993). HB-EGF binds to and gives a mitogenic signal through the EGF receptor and possibly ErbB-4 (Elenius et al. 1997), an EGFR-like subfamily of receptor tyrosine kinases. The soluble form of this growth factor (sHB-EGF) is a potent mitogen for a variety of cell types, including fibroblasts, smooth-muscle cells, and keratinocytes (Higashiyama et al. 1991).

As mentioned already, diphtheria toxin binds to the EGF-like domain of proHB-EGF. Once diphtheria toxin binds to HB-EGF, the diphtheria toxin-bound HB-EGF cannot bind to EGF receptor any more (Mitamura et al. 1995). Therefore, CRM197, a non-toxic mutant of diphtheria toxin, efficiently inhibits the mitogenic activity of human HB-EGF, while it does not inhibit the mitogenic activity of mouse HB-EGF. CRM197 does not inhibit the mitogenic activities of other EGF-receptor ligands, including EGF, TGFα, amphiregulin, and β-cellulin (Mitamura et al. 1995). Thus, CRM197 serves as a specific inhibitor of human-type HB-EGF. The inability of CRM197 to inhibit the mitogenic activities of other EGF-family growth factors indicates that the membrane-anchored form of these growth factors does not serve as a diphtheria-toxin receptor.

2. Juxtacrine Growth Regulator

The membrane-anchored form of HB-EGF (proHB-EGF), i.e. diphtheria-toxin receptor, is not only a precursor of sHB-EGF but is also mitogenically active itself as a growth factor and transmits the mitogenic signal to adjacent cells by interaction with EGF receptors (Higashiyama et al. 1995), a process referred to as juxtacrine stimulation (Massague 1990). Furthermore, in

analogy to diphtheria toxin, DRAP27/CD9 upregulates the juxtacrine activity of the membrane-anchored HB-EGF but does not affect the paracrine activity of sHB-EGF (HIGASHIYAMA et al. 1995). More recently, we have observed that the membrane-anchored HB-EGF shows growth-inhibitory activity by juxtacrine mechanisms in culture conditions under which soluble forms of HB-EGF and EGF stimulate cell growth (MEKADA, submitted). Thus, it is suggested that the soluble form and the membrane-anchored form have different biological activities in cell-growth control. Further studies of the physiological role of HB-EGF in vivo, especially in the membrane-anchored form, remained to be clarified.

A marked feature of proHB-EGF is that it forms a complex with other membrane proteins. In studies of diphtheria-toxin receptor, both DRAP27/CD9 (IWAMOTO et al. 1991) and heparan-sulfate proteoglycan(s) are revealed to bind to proHB-EGF (SHISHIDO et al. 1995). In addition to CD9, proHB-EGF forms a complex with integrin $\alpha 3\beta 1$ (NAKAMURA et al. 1995). Furthermore, other TM4SF-family proteins, including CD63, CD81, and CD82, are associated with the proHB-EGF complex (MEKADA, in preparation). Thus, proHB-EGF forms a large complex which would be involved in growth regulation. Studies using immunofluorescence staining showed that the complex comprised of proHB-EGF, CD9, and integrin $\alpha 3\beta 1$ co-localizes at cell–cell contact sites (NAKAMURA et al. 1995), supporting the notion that proHB-EGF plays a juxtacrine role in intercellular communication.

3. Conversion of the Membrane-Anchored Form to the Soluble Form

The membrane-anchored form of HB-EGF is cleaved at the juxtamembrane domain to yield the soluble HB-EGF. This processing of extracellular domain, or so-called ectodomain shedding, means the conversion of the mode of action of this growth factor from juxtacrine to paracrine. This cleavage is regulated by intracellular signaling. It has previously been shown that Vero cells become resistant to diphtheria toxin upon pretreatment with the phorbol ester phorbol 12-myristate 13-acetate (TPA), an activator of protein kinase C (PKC; SANDVIG and OLSNES 1981; OLSNES et al. 1986). GOISHI et al. (1995) showed that TPA treatment of Vero-H cells (Vero cells over expressing diphtheria-toxin receptor/HB-EGF) induces rapid cleavage of proHB-EGF at a Pro148–Val149 site, to yield soluble HB-EGF. The TPA effects are abrogated by the PKC inhibitors, staurosporin and H7, suggesting the involvement of PKC in this process. Recently, we explored the isotypes of PKC involved in the TPA-induced cleavage of proHB-EGF. Transfection of constitutively active forms of PKCα, PKCδ, or PKCε (and transfection of kinase-negative forms of PKCδ) into Vero-H cells revealed that PKCδ is involved in TPA-induced cleavage of proHB-EGF (IZUMI et al. 1998).

Metalloprotease inhibitors inhibit both the constitutive and TPA-induced cleavage of proHB-EGF (LANZREIN et al. 1995; IZUMI et al. 1998), indicating involvement of metalloprotease-family proteases in this process. To identify

molecules downstream of PKCδ in the TPA-induced cleavage process of proHB-EGF, interaction screening of a cDNA library, using purified PKCδ as a probe, was performed; this identified MDC9, a member of the metalloprotease/disintegrin family, as a specific binding protein for PKCδ. The evidence that overexpression of MDC9 resulted in the cleavage of HB-EGF without TPA and that the MDC9 mutants lacking or deficient in the metalloprotease domain inhibited TPA-induced HB-EGF cleavage indicate that MDC9 is involved in the processing of diphtheria-toxin receptor/HB-EGF and that a direct interaction between PKCδ and MDC9 is involved in the regulated cleavage (Izumi et al. 1998). In other words, conversion of HB-EGF from the membrane-anchored form to the soluble form is regulated by an inside-out signaling mechanism of PKC. The regulated shedding of membrane proteins has also been shown for a number of other membrane proteins, including β-amyloid precursor protein, TGFα, interleukin-6, and receptor-like protein tyrosine phosphatases (Pandiella and Massague 1991a, 1991b; Arribas et al. 1996). The analysis of the cleavage mechanism of diphtheria-toxin receptor/proHB-EGF would be helpful in understanding this interesting mechanism.

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CHAPTER 4

Pseudomonas aeruginosa Exotoxin A: Structure/Function, Production, and Intoxication of Eukaryotic Cells

S.E.H. West

A. Introduction

Exotoxin A (ETA) is the most toxic of the numerous extracellular proteins (LasA and LasB elastases, alkaline protease, protease IV, hemolytic and non-hemolytic phospholipase C, exoenzyme S, and cytotoxin) produced by the opportunistic pathogen *Pseudomonas aeruginosa*. ETA belongs to a class of secreted bacterial toxins that transfer the adenosine diphosphate (ADP)–ribose moiety of nicotinamide adenine dinucleotide cation (NAD^+) to specific target proteins within eukaryotic cells (Krueger and Barbieri 1995). ETA, like many of these toxins, conforms to a simple A–B structure–function model in which the A domain possesses enzymatic activity and the B domain binds to a specific receptor on the surface of target cells.

ETA was first characterized by P.V. Liu in 1966 as a heat-labile, trypsin-sensitive protein exotoxin in culture supernatants of *P. aeruginosa* (Liu 1966). Early studies to determine the mode of action of ETA demonstrated that purified ETA reduced protein synthesis in the livers of ETA-treated animals (Pavlovskis et al. 1974). The ability of ETA to inhibit protein synthesis was confirmed by Iglewski and Kabat (1975), who demonstrated that ETA catalyzes the transfer of the ADP–ribose moiety of NAD^+ to elongation factor 2 in a rabbit-reticulocyte cell-free lysate. Elongation factor 2 is responsible for the guanosine triphosphate-hydrolysis-dependent translocation of eukaryotic ribosomes during protein synthesis. This is the same enzymatic reaction that is catalyzed by diphtheria toxin (Iglewski et al. 1977). Both toxins modify a unique, post-translationally modified histidine residue called diphthamide (Van Ness et al. 1980). Inactivation of diphthamide results in cessation of protein synthesis, which leads to cell death. In addition to ADP–ribosyltransferase activity, ETA also possesses NAD^+-glycohydrolase activity in the absence of elongation factor 2 (Chung and Collier 1977a).

Even though these two toxins catalyze the same enzymatic reaction, there are major differences in their structures and modes of action. Both ETA and diphtheria toxin are synthesized as a single polypeptide chain; however, the domain responsible for enzymatic activity is located in the C-terminus of ETA but resides in the N-terminus of diphtheria toxin (Gray et al. 1984). These toxins are toxic for different cell lines (Middlebrook and Dorland 1977; Vasil and Iglewski 1978). For example, mouse cells are

more resistant to ETA than to diphtheria toxin. This observation indicates that they bind to different receptors and that the binding domains of these two proteins are distinct. The amino acid sequences of these two proteins are highly divergent (Gray et al. 1984); this difference is confirmed by their limited immunological cross-reactivity (Sadoff et al. 1982). However, recent crystallographic studies of the enzymatic regions of several ADP–ribosyltransferases indicate that the essential amino acids in the NAD-binding sites of ETA and diphtheria toxin are conserved and that the three-dimensional structures of the enzymatic domains are superimposable (Carroll and Collier 1988; Domenighi et al. 1991).

ETA has provided a unique opportunity for study of toxin-domain structure as it relates to specific functional regions of the molecule. This information is being used to develop recombinant immunotoxins designed for the selective killing of cancer cells (Fitzgerald 1996; Pastan et al. 1996; Pastan 1997). Studies on the regulation of ETA production have provided insight into the global mechanisms involved in sensing and adapting to specific growth conditions. This review will attempt to summarize the molecular aspects of ETA structure and function, regulation of ETA production, secretion of ETA from the bacterial cell, and intoxication of eukaryotic cells by ETA.

I. Basic Structure

ETA was the first ADP–ribosyltransferase to have its X-ray-crystallographic structure determined to the 3.0-Å level of resolution (Allured et al. 1986). It is synthesized as a 638-amino-acid precursor which is processed during secretion to a 613-amino-acid, enzymatically inactive proenzyme form (Gray et al. 1984). ETA contains eight cysteine residues, which form sequential disulfide bonds. X-ray-crystallographic analysis of the proenzyme form of ETA revealed that the molecule consists of three distinct structural domains (Allured et al. 1986; Guidi-Rontani and Collier 1987). Domain I encompasses amino acids 1–252 (Ia) and 365–399 (Ib) arrayed in 17 antiparallel β-strands. Domain II includes residues 253–364 and is composed of six α-helices; helices A and B are linked by a disulfide bond. Domain III is comprised of amino acids 400–613, contains an extended cleft or fold, and has a less regular secondary structure. Deletion and mutation analysis of the ETA molecule has revealed that domain Ia contains signals that direct the secretion of ETA from the bacterial cell; it is also involved in the binding of ETA to a specific eukaryotic receptor. Domain II is involved in the internalization and translocation of ETA to the cytosol of the eukaryotic cell, and domain III possesses the ADP–ribosyltansferase and NAD-glycohydrolase activities (Chung and Collier 1977b; Vasil et al. 1977; Lory and Collier 1980; Guidi-Rontani and Collier 1987; Hwang et al. 1987; Guidi-Rontani 1991; Lu et al. 1993; Lu and Lory 1996). The function of domain Ib is unknown, and this region can be deleted without loss of cytotoxicity (Kihara and Pastan 1994).

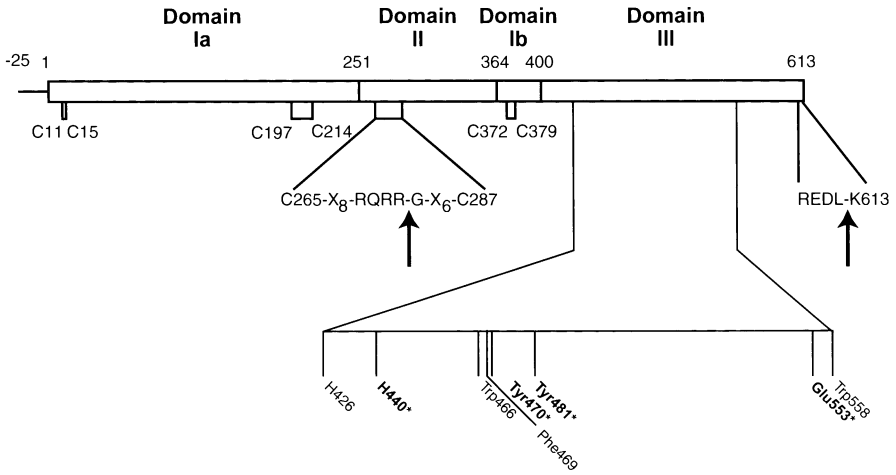

Fig. 1. Structure–function relationships of ETA. Structural domains are designated Ia, Ib, II, and III; numbers refer to amino acid residues (ALLURED et al. 1986; GUIDI-RONTANI and COLLIER 1987). Disulfide bonds are indicated by *bridges* below the ETA molecule and are labeled, along with their cysteine residues. Domain Ia is involved in secretion of the toxin from the bacterial cell and binding to the α_2-macroglobulin/low-density lipoprotein-receptor-related protein. Domain II is involved in the internalization and translocation of ETA, and domain III possesses enzymatic activity (CHUNG and COLLIER 1977b; VASIL et al. 1977; LORY and COLLIER 1980; GUIDI-RONTANI and COLLIER 1987; HWANG et al. 1987; GUIDI-RONTANI 1991; LU et al. 1993; LU and LORY 1996). Sites of proteolytic cleavage are indicated by a *bold arrow*. The endopeptidase furin cleaves ETA between Arg279 and Gly280 to yield a 28-kDa, N-terminal fragment containing domain Ia and part of domain II and a 37-kDa, enzymatically active fragment containing part of domain II and domains Ib and III (OGATA et al. 1990, 1992; CHIRON et al. 1994, INOCENCIO et al. 1994; GORDON et al. 1995). The terminal lysine residue is removed by a serum carboxypeptidase (HESSLER and KREITMAN 1997). Amino acid residues conserved among ADP–ribosyltransferases are shown, and those implicated in enzymatic activity are indicated by an *asterisk* (DOMENIGHINI et al. 1991, 1996)

Before it can catalyze the ADP–ribosylation of elongation factor 2, the proenzyme form of ETA must undergo a conformational change to remove the steric constraints imposed on domain III by the other parts of the molecule (VASIL et al. 1977). In vitro, the activation of ETA can be achieved either by treatment with denaturing and reducing agents, such as urea and dithiothreitol, or by proteolytic cleavage to yield an enzymatically active fragment (CHUNG and COLLIER 1977b; VASIL et al. 1977; LEPPLA et al. 1978; LORY and COLLIER 1980). In vivo, ETA is activated by proteolytic cleavage and by breakage of a disulfide bond to yield an enzymatically active 37-kDa fragment (CHIRON et al. 1994). Activation of the proenzyme form is not necessary for NAD-glycohydrolase activity (CHUNG and COLLIER 1977b). Figure 1 depicts the structure–function relationships of ETA.

II. Role of ETA in Disease

P. aeruginosa is a metabolically diverse, obligate respiring, motile, Gram-negative microorganism which has a predilection for moist environments. It is primarily found in water and soil and on vegetation. *P. aeruginosa* infects injured, immunodeficient, or otherwise compromised patients; it rarely causes disease in a healthy person. For this reason, *P. aeruginosa* is considered to be an opportunistic pathogen. In compromised patients, *P. aeruginosa* causes acute infections, such as corneal ulcers, otitis media, septicemia, acute pneumonia, systemic infections associated with severe burns or neutropenia, and a chronic lung infection in cystic-fibrosis patients (Fergie et al. 1994; Richard et al. 1994; Mendelson et al. 1994; Dunnard and Wunderink 1995; Bergen and Shelhamer 1996; Brewer et al. 1996). *P. aeruginosa* lung infections are the eventual cause of death in 90% of cystic-fibrosis patients (Govan and Deretic 1996). *P. aeruginosa* is the most common Gram-negative bacterium found in nosocomial infections, which are often life threatening because of the innate resistance of this organism to most antimicrobial agents (Pollack 1995; Kluytmans 1997; Wiblin 1997; Gordon et al. 1998).

A considerable amount of evidence has been generated, indicating that ETA plays a significant role in infections caused by *P. aeruginosa*. ETA is produced by 95% of all clinical isolates of *P. aeruginosa* (Bjorn et al. 1977; Vasil et al. 1986). Saelinger and colleagues (1977, 1985, 1987) demonstrated that ETA is produced by *P. aeruginosa* multiplying at burn sites. Early in these infections, ETA enters the bloodstream and initially blocks protein synthesis in the liver (Saelinger et al. 1977); later, the spleen, heart, kidneys, and lungs are affected (Snell et al. 1978). Pavlovskis et al. (1977) demonstrated that the levels of active elongation factor 2 were reduced 70–90% in the livers of *P. aeruginosa*-infected mice; other organs were affected to a lesser extent. Thus, the liver appears to be the primary organ that is affected by ETA in septicemic burn patients. Administration of anti-ETA antiserum to burned mice infected with *P. aeruginosa* PA103, an ETA-hyperproducing strain which produces low levels of protease, significantly increased survival (Pavlovskis et al. 1997). This result suggested that ETA contributed to the lethality of *P. aeruginosa* in a burn-infection model. The role of ETA in septicemia, bacteremic pneumonia, and burn-wound infections has been established through both the protective effects of anti-ETA antiserum in an experimental burn model and the presence of anti-ETA antibodies in the sera of infected patients (Pollack et al. 1976, 1979, 1983; Pavloskis et al. 1977; Saelinger et al. 1977; Cross et al. 1980).

The precise role of ETA in the chronic lung infections of cystic-fibrosis patients is unclear, since evidence of direct tissue damage by ETA is lacking. However, ETA is a polyclonal T cell mitogen, or "super antigen" which, at subcytotoxic levels, generates cytotoxic T lymphocytes and induces interleukin-1 production from peritoneal macrophages (Zehavi-Wilner 1988; Misfeldt 1990; Misfeldt et al. 1990; Legaard et al. 1992; Dixon and Misfeldt 1994). These data suggest that ETA may be responsible for eliciting an inappropri-

ate inflammatory response that contributes to host-tissue damage. Small amounts of ETA have been found in the sputa of cystic-fibrosis patients during periods of exacerbated lung disease (GRIMWOOD et al. 1993; JAFFAR-BANDJEE et al. 1995). Storey and colleagues have also detected the presence of ETA transcripts in a majority of patients and, in some patients, they detected high levels of ETA messenger RNA (mRNA) (STOREY et al. 1992; RAVIO et al. 1994). Significantly higher levels of anti-ETA antibodies were found in the sera and sputum of cystic-fibrosis patients than in control groups of *P. aeruginosa*-infected patients (JAGGER et al. 1982; CUKOR et al. 1983; GRANSTROM et al. 1984; Moss et al. 1986). Thus, the prognosis of cystic-fibrosis patients is inversely correlated with high levels of circulating immune complexes as a result of an immunoglobulin-G response to ETA.

In several animal models mimicking the various types of *P. aeruginosa* infections, the virulence of ETA-deficient mutants was found to be significantly less than that of the wild-type parental strain, thus strengthening the evidence that ETA plays a significant role in the virulence of *P. aeruginosa*. These models include acute and chronic lung infections, bacteremia associated with severe burn infections, and corneal infections (STIERITZ and HOLDER 1975; OHMAN et al. 1980; WOODS et al. 1982; BLACKWOOD et al. 1983; NICAS and IGLEWSKI 1985).

B. Production of ETA by the Bacterial Cell

I. Characterization of the *toxa* Structural Gene

The gene encoding the 638-amino-acid ETA precursor was first cloned by GRAY et al. (1984) and was designated *toxA*. VASIL et al. (1986) found that the *toxA* gene is present in 95% of *P. aeruginosa* strains as a single copy per genome. *toxA* is transcribed as a monocistronic message from two apparent transcriptional start sites (GRAY et al. 1984; GRANT and VASIL 1986). The most upstream start site, designated S1a and located 89 bp 5′ of the translational start, appears to be used more frequently than the downstream site, designated S1b, which is located 62 bp 5′ of the translational start. When the *toxA* transcriptional start sites are aligned, the regions extending 45–46 bp upstream of each start site are 58% identical (WEST et al. 1994a). Thus, the *toxA* promoter region appears to consist of two similar, overlapping promoters. The *toxA* promoter region shares no sequence similarity with other known *P. aeruginosa* promoters or with the consensus sequences for *Escherichia coli* promoters.

II. Environmental and Temporal Signals Affecting ETA Production

Several external stimuli that regulate ETA synthesis have been identified and include iron limitation, the presence or absence of divalent cations and various metals, glycerol, and temperature (LIU 1973; LORY 1986; BLUMENTALS et al.

1987). Lory (1986) showed that concentrations of iron above 2 μM repress *toxA*-mRNA production in a dose-dependent manner; at concentrations above 30 μM, production of both *toxA* mRNA and extracellular ETA are repressed approximately 95%. Although not as well characterized as the repression of ETA synthesis by iron, the addition of either 3.5% glycerol or 500 μM Ca^{2+} to the culture medium results in an approximately threefold increase in the quantity of *toxA* mRNA (Blumentals et al. 1987). In contrast, the addition of 500 μM Mn^{2+} to the culture medium decreased production of *toxA* mRNA by 63% (Blumentals et al. 1987). Liu (1973) initially demonstrated that temperature inversely regulates ETA production; other laboratories have characterized this effect and found that increasing the temperature from 27°C to 37°C resulted in 70% and 84% decreases in ETA production in the *P. aeruginosa* strains PA103 and PAO1, respectively (Vasil, personal communication; West and Runyen-Janecky, unpublished). These results suggest that the effect of temperature on ETA production is as great as the effect of iron.

ETA production is also regulated in a temporal manner. Frank and Iglewski (1988) found that, under iron-limiting conditions, *toxA* is transcribed in a biphasic manner. During the early phase of logarithmic growth, a small peak of *toxA* mRNA accumulates, which corresponds to the production of a small quantity of intracellular toxin. A second and greater accumulation of *toxA* mRNA, which is associated with the maximal production of extracellular ETA, peaks as the cells enter the stationary phase of growth. This second phase of *toxA* transcription is stringently repressed by iron (Frank et al. 1989). The mechanism responsible for the temporal regulation of ETA production has not been identified.

III. Regulation of ETA Production

Several *trans*-acting regulatory factors, including the *P. aeruginosa* cyclic adenosine monophosphate (AMP)-receptor-protein (CAP) homologue Vfr, the novel proteins RegA and RegB, the quorum-sensing regulator LasR, the iron-responsive transcriptional repressor Fur, the PtxR protein, and the alternative σ factor PvdS, influence *toxA* expression (Hedstrom et al. 1986; Hindahl 1988; Wick et al. 1990; Gambello et al. 1993; West et al. 1994b; Oschner et al. 1995, 1996; Hamood et al. 1996). Several of these regulatory factors also affect the production of other *P. aeruginosa* virulence factors. Thus, the regulation of ETA synthesis appears to be intricately interwoven into the regulatory networks that control production of the LasB and LasA elastases, alkaline protease, rhamnolipid, and the siderophore pyoverdin. This control of ETA production through inter-connected regulatory circuits is in contrast to the coordinate regulation of multiple virulence factors by a single regulatory system in response to one or more environmental signals, as occurs in *Vibrio cholerae* and *Bordetella pertusssis*. The way in which the various proteins that modulate ETA expression interact is depicted in Fig. 2.

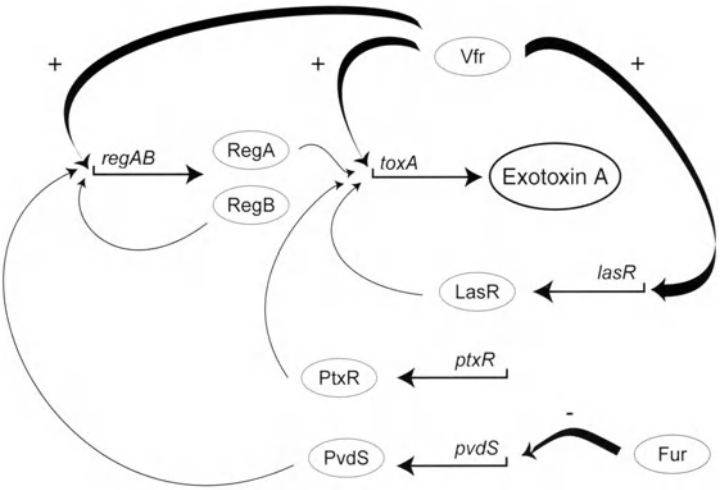

Fig. 2. Regulation of ETA production. Regulatory proteins are shown as *ellipses*. Genes are indicated by *italics* and are underlined with an *arrow*. *Bold arrows* indicate regulatory relationships which have been confirmed by gel-shift analysis and/or DNaseI footprinting; other interactions are based solely on genetic data. Positive interactions are indicated by +; negative interactions are indicated by –

Of the numerous regulatory proteins that influence ETA production, Vfr is the only one that has been shown by DNase-I footprinting to interact directly with the *toxA* promoter; Vfr also binds to the *regAB* and *lasR* promoters (Runyen-Janecky et al. 1996; Albus et al. 1997). Vfr is the *P. aeruginosa* homologue of *E. coli* CAP, which regulates the transcription of numerous genes in *E. coli*, most notably those involved in the utilization of various carbon sources (West et al., 1994b). The derived amino acid sequences of these two proteins are 67% identical and 91% similar. Unlike CAP, Vfr is not involved in catabolite-repression control (MacGregor et al. 1995). In addition to *toxA*, the genes that are activated by Vfr include the ETA regulators *regA* and *regB*, *lasR*, which encodes a quorum-sensor regulator, and the gene encoding protease IV or its regulator (West et al. 1994b; Albus et al. 1997; Engel et al. 1998). ETA production is reduced 99% in a *vfr* mutant (West et al. 1994a). In a DNase-I footprinting assay, Vfr protected the sequence 5′ACCACTCTGCAATCCAGTTCATAAA3′ centered 70 bp 5′ of the S1a start site in the *toxA* promoter (Runyen-Janecky et al. 1996).

The *regA* gene was identified by its ability to complement in *trans* the ETA-deficient phenotype of the nitrosoguanidine-generated mutant PA103-29 (Hedstrom et al. 1986; Hindahl et al. 1988). *regA* Encodes a 29-kDa protein which has been localized to the inner membrane. The deduced amino acid sequence of *regA* shares no homology with any known proteins; therefore, it has been difficult to ascribe a function to RegA. Hamood and Iglewski (1990) postulated that RegA is a transcriptional activator, because it contains

a helix–turn–helix motif characteristic of DNA-binding proteins. However, they were unable to demonstrate that RegA could bind the *toxA* promoter. WALKER et al. (1994) demonstrated that purified RegA stimulated a 15-fold increase in *in vitro* transcription of *toxA* by purified *P. aeruginosa* RNA polymerase; addition of *P. aeruginosa* cell extracts resulted in another threefold enhancement of transcription. Because RegA co-purified with RNA polymerase on a gel-filtration column and because an RNA polymerase/RegA complex could be immunoprecipitated with RegA-specific antisera, WALKER et al. (1994) proposed that RegA and RNA polymerase interact.

The *regB* gene product is responsible for hyperproduction of ETA in strain PA103 (WICK et al. 1990). The *regB* gene is located immediately downstream of *regA* and has no apparent ribosome-binding site. There does not appear to be an absolute requirement of RegB for ETA production.

PvdS is an alternative σ factor that is required for production of both ETA and the siderophore pyoverdin (OCHSNER et al. 1996). In a *pvdS*-deletion mutant, there is a 95% reduction in the amount of ETA produced in response to iron starvation. In addition, both *toxA* and *regAB* mRNA were not detected. When the *pvdS* gene was overexpressed under the control of the inducible *tac* promoter, high levels of ETA were produced independent of the iron concentration of the culture medium. ETA was not produced when *pvdS* was overexpressed in a *regA*-deletion mutant. This finding suggests that PvdS is required for activation of the *regAB* promoters (OSCHNER et al. 1996). It is not clear whether PvdS directly regulates both *toxA* and *regAB* or indirectly regulates *toxA* through either RegA or an unidentified regulatory protein.

In *E. coli* and other enteric microorganisms, the Fur protein represses the expression of numerous iron-regulated genes, most notably those involved in iron acquisition. This repression occurs when a Fe^{2+}/Fur complex binds to the "Fur box" present in the promoters of *fur*-regulated genes. OCHSNER et al. (1995) demonstrated that purified Fur protein bound DNA fragments containing the *pvdS* promoter but not the *toxA* or *regAB* promoters. Thus, in response to changing iron levels, *P. aeruginosa* Fur appears to regulate *toxA* production indirectly by repressing expression of *pvdS* (OCHSNER et al. 1996).

LasR is a quorum-sensing regulator which enhances *toxA* expression and also activates transcription of the elastase structural genes *lasA* and *lasB* and the alkaline-protease structural gene *apr* (GAMBELLO et al. 1993). In a *P. aeruginosa* PAO1 *lasR* mutant, ETA expression was reduced by 33%; the addition of *lasR* on a multicopy plasmid increased ETA levels threefold. This effect was not mediated through RegA, as LasR did not affect transcription of *regAB*.

PtxR is a 35-kDa protein which shares sequence similarity with the LysR family of transcriptional activators (HAMOOD et al. 1996). In an iron-deficient medium, the presence of a plasmid carrying *ptxR* increased ETA synthesis by four- to fivefold. In an in vitro, constructed *ptxR*-deletion mutant, ETA production was reduced 40–50%; however, upon extensive subculturing, the level of ETA produced by the *ptxR* mutant returned to parental levels.

In summary, production of ETA is a finely tuned event that occurs in response to one or more environmental signals and is mediated by multiple proteins which also function in the regulatory circuits that control expression of other *P. aeruginosa* virulence factors, such as elastase, alkaline protease, and siderophores.

IV. Secretion from the Bacterial Cell

In gram-negative bacteria, secretion of an extracellular protein requires that the protein be transported across two hydrophobic lipid bilayers and an aqueous periplasmic space. ETA and several other *P. aeruginosa* exoproteins are secreted from the bacterial cell by the type-II general secretion pathway (Pugsley 1993). In this process, the secreted proteins are initially translocated from the inner membrane into the periplasm and then are translocated across the outer membrane. To aid this process, specific targeting signals or structures that are present in the ETA molecule direct its secretion to the periplasm and to the extracellular milieu.

ETA possesses a 25-amino-acid N-terminal leader peptide which directs the ETA protein across the cytoplasmic or inner membrane (Gray et al. 1984; Douglas et al. 1987). This leader peptide is similar to other bacterial leader sequences in that it contains basic amino acid residues at the N-terminus (histidine residues at positions –24 and –20), a 13-amino-acid central core region that contains ten hydrophobic residues, and a consensus Ala–(Ser)–Ala leader peptidase-recognition site. Douglas et al. (1987) demonstrated that enzymatically active ETA is secreted into the periplasm of *E. coli* and that a mutation in the *secA* gene blocked secretion, processing, and conversion of the protein to a fully toxic conformation. Thus, the translocation of ETA across the inner membrane is presumably mediated by a secretion apparatus similar to that encoded by the *sec* genes of *E. coli*. However, a *sec*-like secretion apparatus has not been described in *P. aeruginosa*.

In *P. aeruginosa*, after removal of the signal peptide, ETA is released into the periplasm without the accumulation of a significant periplasmic pool (Lory et al. 1983). Initially, this failure to detect ETA in the periplasm led to the hypothesis that ETA is secreted by a novel mechanism in which it is inserted into the inner membrane and is transported to the outer membrane through zones of adhesion between the two membranes. However, Lu et al. (1993) demonstrated that mutagenesis of amino acid residues at the extreme N-terminus results in transient accumulation of ETA in the periplasm. Specifically, if lysine and glutamine were substituted for the +2 and +3 glutamic acid residues, the mutant ETA proteins accumulated in the periplasm and membrane fractions before their eventual transport into the extracellular milieu. This study provided evidence that ETA is secreted by a two-step process.

The terminal branch of the *P. aeruginosa* general-secretory pathway is comprised of at least 12 *xcp* genes, which are similar to the *Klebsiella oxytoca pul* genes that are involved in the specific secretion of pullulanase (Bally et al. 1992; Nunn and Lory 1992; Akrim et al. 1993; Pugsley 1993). The XcpQ,

XcpT, XcpU, XcpV, and XcpW proteins comprise the secretory apparatus; XcpQ is the only Xcp protein that is located in the outer membrane. It has been proposed that these proteins recognize a specific conformational structure on the surface of the folded ETA molecule. The XcpP, XcpX, XcpY, and XcpZ proteins are inner-membrane components; their function has not been determined (Bleves et al. 1996). The XcpA, XcpR, and XcpS proteins are required for the assembly of the secretion apparatus. Specifically, XcpR contains an adenosine triphosphate (ATP)-binding site and presumably hydrolyzes ATP to provide energy for the translocation of secreted proteins across the outer membrane (Turner et al. 1993). XcpA, also identified as PilD, is a leader peptidase and *N*-methyl transferase that is responsible for the cleavage of the type IV pilin-like leader peptides of the XcpT, XcpU, XcpV, and XcpW proteins (Strom et al. 1991; Bally et al. 1992). This protein is also required for the processing of several proteins that are involved in the assembly or are structural components of the type-IV pili produced by *P. aeruginosa*. If the leader peptide is not cleaved from XcpT, XcpU, XcpV, and XcpW, the secretory apparatus does not form properly, and ETA and other secreted proteins accumulate in the periplasm (Strom et al. 1991). In a *pilD* (*xcpA*) mutant, ETA accumulated in the periplasm; this protein was fully mature in size, contained all cysteines in disulfide bonds and was toxic in a tissue-culture cytotoxicity assay. This result suggests that ETA is folded into its native conformation before translocation across the outer membrane.

For transport across the outer membrane, a protein must possess a specific signal that distinguishes it from those proteins that remain in the periplasm. Using ETA-β-lactamase hybrid proteins, Lu and Lory (1996) demonstrated that residues 60–120 within ETA domain Ia can target the secretion of mature β-lactamase across the outer membrane of *P. aeruginosa*. Normally, β-lactamase is only secreted to the periplasm of *P. aeruginosa*. In this region of ETA, there are three anti-parallel β-strands which appear as a protruding knob; no other recognizable structural motifs are found within this region. Lu and Lory (1996) hypothesized that this knob is the signal recognized by the terminal branch of the *P. aeruginosa* general-secretory apparatus for translocation of ETA across the outer membrane.

C. Intoxication of Eukaryotic Cells

Intoxication of a eukaryotic cell by ETA requires that ETA be transported to the cytosol, where elongation factor 2 is located. If ETA is not delivered to the cytosol, the target cell will escape intoxication. The intoxication process involves the following steps: (1) binding of ETA to a specific receptor, (2) internalization by receptor-mediated endocytosis, (3) activation of the protein by proteolytic cleavage and a conformational change, (4) removal of the terminal lysine residue and movement from the trans-Golgi to the endoplasmic reticulum, and

(5) translocation to the cytosol, where (6) ETA ADP–ribosylates elongation factor 2. The steps subsequent to endocytosis, which result in the transport of ETA to the cytosol, are not completely understood. Figure 3 depicts the steps involved in the intoxication of eukaryotic cells by ETA.

I. Binding to a Specific Receptor on the Eukaryotic Cell Surface and Internalization by Receptor-Mediated Endocytosis

The initial step in the intoxication of the eukaryotic cell by ETA is binding to a specific receptor on the surface of susceptible cells. Saelinger and colleagues have purified and characterized a protein (from mouse LM fibroblasts and from mouse liver) which binds ETA (Forristal et al. 1991; Thompson et al. 1991). This protein was shown to be immunologically and functionally identical to the heavy chain of the multi-functional receptor known as the α_2-macroglobulin/low-density lipoprotein-receptor-related protein (LRP) on the cell surface (Kounnas et al. 1992). LRP is widely distributed on many types of tissues and is present at high levels on the surfaces of fibroblasts and hepatocytes. LRP mediates the clearance of proteases, protease-inhibitor complexes, and various ligands associated with lipid metabolism. As further confirmation that LRP is the ETA receptor, Willnow and Herz (1994) and Fitzgerald et al. (1995), by selecting for resistance to ETA, have isolated mutant cell lines that are deficient in the surface expression of LRP. Avramoglu et al. (1998) demonstrated that transfection with expression plasmids carrying full-length chicken LRP restored sensitivity to ETA in a mutant Chinese hamster ovary cell line that lacked expression of endogenous LRP. Thus, ETA appears to bind to mammalian cells by utilizing an "opportunistic" receptor.

Mucci et al. (1995) proposed that the level of LRP and/or the level of receptor-related protein (RAP) on the surfaces of susceptible cells modulates susceptibility to ETA. RAP is a 39-kDa protein which co-purifies with LRP and blocks the binding of ETA and other ligands to LRP. Cells expressing low levels of LRP were 200-fold more resistant to ETA than sensitive cells, and cells expressing high levels of both LRP and RAP were also resistant to ETA. In the latter case, RAP presumably prevents ETA from binding to LRP.

The specific amino acids in domain Ia of ETA that interact with LRP have not been identified. However, ETA binding and/or cytotoxicity were abolished or greatly diminished when Lys57 was mutated to Glu or Gly (Jinno et al. 1988) or when a dipeptide was inserted between positions 60 and 61 (Chaudry et al. 1989).

Once bound to LRP, ETA enters mammalian cells by receptor-mediated endocytosis, the process by which many growth factors, hormones, and transport proteins enter cells (Morris 1990). Using electron microscopy, several investigators have followed the entry of ETA into mouse LM fibroblasts (Morris et al. 1983; Morris and Saelinger 1985, 1986; Saelinger et al. 1985, 1987). In these studies, biotinylated ETA was detected with avidin–gold colloids. Following binding of ETA to LRP, the LRP–ETA complex moved to

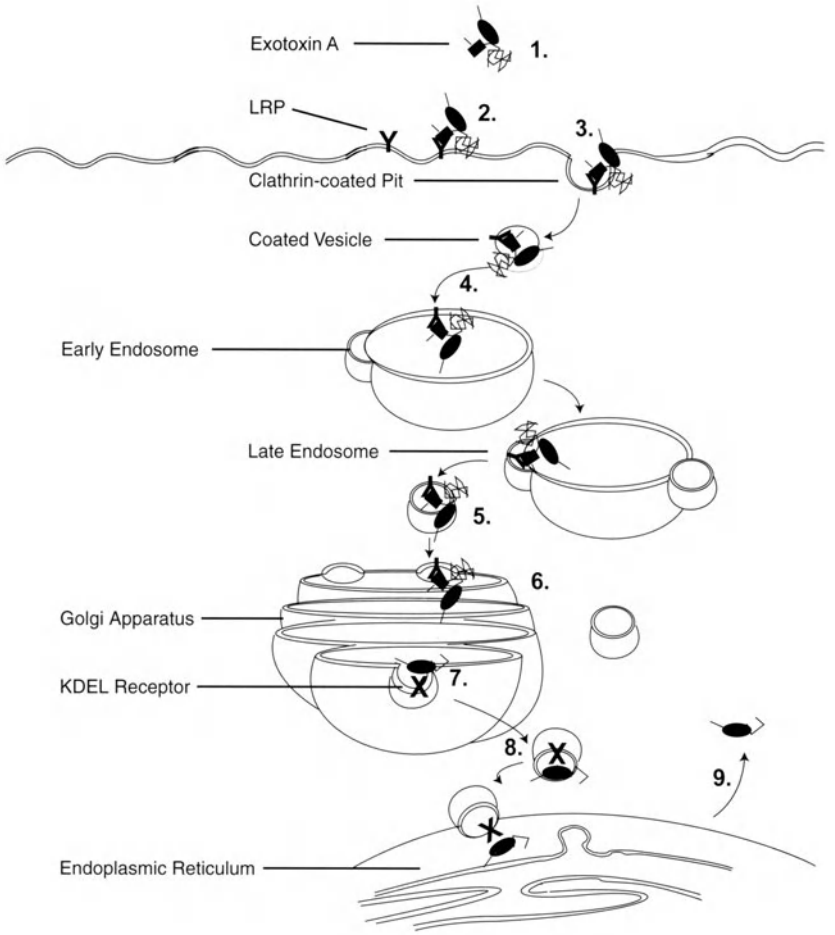

Fig. 3. Intoxication of eukaryotic cells by ETA. The following steps have been proposed as the mechanism by which ETA intoxicates eukaryotic cells. (1) Removal of the terminal lysine residue from ETA (Chaudhary et al. 1990). (2) Binding of ETA to the α_2-macroglobulin/low-density lipoprotein-receptor-related protein (LRP) (Forristal et al. 1991; Thompson et al. 1991). (3) Movement of the ETA/LRP complex to clathrin-coated pits (Morris et al. 1983; Morris and Saelinger 1985, 1986; Saelinger et al. 1985, 1987). (4) Fusion of the coated pit with an endosome. (5) Translocation to the Golgi apparatus. (6) Cleavage of ETA by the eukaryotic endopeptidase furin, and reduction of the disulfide bond between Cys265 and Cys287 (Ogata et al. 1990, 1992; Chiron et al. 1994; Inocencio et al. 1994; Gordon et al. 1995). (7) Binding to the KDEL receptor. (8) Translocation to the endoplasmic reticulum. (9) Release of the enzymatically active 37-kDa ETA fragment into the cytosol, where it can catalyze the adenosine-diphosphate–ribosylation of elongation factor 2 (Iglewski and Kabat 1975; Chung and Collier 1977a). Not all of these steps have been characterized

clathrin-coated pits, which were pinched off to become coated vesicles. At 37°C, this step occurred 30s to 2.5min after the addition of the biotinylated ETA to the LM cells. Within 10min, ETA was found in endosomes, the result of fusion of the coated vesicle with a non-coated vesicle. After 10–20min, the toxin appeared in the trans-Golgi region and, after 30-60min, it could be detected in the lysosomal compartment. This process could be blocked by agents, such as methylamine, ammonium chloride, and chloroquine, which prevent receptor-mediated endocytosis (FITZGERALD et al. 1980; MORRIS et al. 1983); the process could also be altered by reduced temperatures (MORRIS and SAELINGER 1986).

II. Activation by Proteolytic Cleavage and/or a Conformational Change

Several investigators (OGATA et al. 1990, 1992; CHIRON et al. 1994; INOCENCIO et al. 1994; GORDON et al. 1995) recently demonstrated that the trans-Golgi endopeptidase furin cleaves ETA to generate an N-terminal 28-kDa fragment and an enzymatically active 37-kDa C-terminal fragment. Furin is a subtilisin-like Ca^{2+}-dependent endoprotease which has been localized to the trans-Golgi network and also to the cell surface (NAKAYAMA 1997). Cleavage of ETA occurred as early as 10min after internalization; this timing corresponds to arrival of the ETA molecule in the trans-Golgi (OGATA et al. 1990).

Furin recognizes the sequence RXK/RR and cleaves ETA at the C-terminal side of the sequence RHRQPR (amino acids 276–279), which is located within a loop in domain II formed by a disulfide bond (Cys-265 to Cys-287; CHIRON et al. 1994). ETA molecules containing mutations that change Arg-276 and Arg-279 to Gly are resistant to cleavage and are approximately 500-fold less cytotoxic. Mutations that change Cys-265 and Cys-287 to Ser or Ala decrease cytotoxic activity about tenfold (MADSHUS and COLLIER 1989); these mutations would prevent formation of the arginine-rich loop containing the furin cleavage site.

To confirm that furin is the protease responsible for cleaving ETA in vivo, MOEHRING et al. (1993) transfected a complementary DNA (cDNA) encoding furin into an ETA-resistant cell line. This cell line, which is deficient in the cleavage of ETA and, therefore, is resistant to ETA, contains a defect in the gene encoding furin (INOCENCIO et al. 1994). Transfection with the furin cDNA rendered these cells sensitive to ETA (MOEHRING et al. 1993).

After ETA is cleaved by furin, the 28-kDa N-terminal fragment and the 37-kDa C-terminal fragment remain linked by a disulfide bond between Cys265 and Cys287 (OGATA et al. 1990). This bond is subsequently reduced (by an unknown mechanism) to release the two fragments. Fractionation of cells exposed to radiolabeled ETA demonstrated that the 37-kDa fragment was enriched in the cytosol, indicating that this fragment had been translocated to the cytosol. The membrane fraction contained the 37-kDa fragment, still linked by a disulfide bond to the 28-kDa fragment.

III. Removal of the Terminal Lysine and Translocation into the Cytosol

The presence of a KDEL-like sequence (REDLK) at the C-terminus of ETA suggests that the 37-kDa fragment is transported from the trans-Golgi to the endoplasmic reticulum before translocation into the cytosol (CHAUDHARY et al. 1990). Newly synthesized proteins that are destined to remain in the endoplasmic reticulum have the sequence KDEL at their C-terminus. This sequence mediates their binding to the KDEL receptor and their subsequent return to the endoplasmic reticulum. Removal of the ETA terminal lysine residue allowed ETA to bind to the KDEL receptor; removal of additional residues blocked cytotoxicity, presumably by preventing binding of ETA to the KDEL receptor, thus preventing translocation of ETA to the endoplasmic reticulum (CHAUDHARY et al. 1990). Removal of these residues did not block *in vitro* ADP–ribosyltransferase activity. The native 37-kDa fragment binds to the KDEL receptor with approximately 1% of the affinity of mutant proteins containing a KDEL-receptor-binding consensus sequence at their C-teminus (KREITMAN and PASTAN 1995). HESSLER and KREITMAN (1997) have shown that the terminal lysine residue of ETA can be removed by a carboxypeptidase present in serum. This occurred when ETA was incubated with cells in medium containing serum or when ETA was incubated in plasma without cells. Thus, this step appears to occur before ETA enters the eukaryotic cell and is transported to the trans-Golgi.

IV. ADP–Ribosylation of Elongation Factor 2

In the cytosol, the 37-kDa fragment of ETA catalyzes the transfer of the ADP–ribose moiety of NAD^+ to diphthamide, a unique residue present in elongation factor 2. NAD^+ initially binds to the 37-kDa fragment of ETA to form a binary complex, which then binds to elongation factor 2 (CHUNG and COLLIER 1977a). To define the site of NAD binding and the mechanism of activation of ETA, LI et al. (1995, 1996) determined, at 2.3-Å resolution, the crystal structure of domain III (amino acids 400–613) complexed with either the hydrolysis-resistant NAD analog β-methylene-thiazole-4-carboxamide adenine dinucleotide (β-TAD) or the products of NAD hydrolysis (AMP and nicotinamide). Unlike the proenzyme form of ETA, domain III, which does not contain any disulfide bonds, does not need to be activated by treatment with denaturing agents to express ADP–ribosyltransferase activity. Thus, the results obtained with this study should yield a conformation that resembles the binary complex of NAD^+ bound to an enzymatically active fragment of ETA. LI et al. (1995, 1996) found that the site for NAD^+ binding and enzymatic activity resided within the extended cleft previously identified by ALLURED et al. (1984) and BRANDHUBER et al. (1988). This cleft consists of two approximately orthogonal, antiparallel β-sheets flanked by several helices. The only significant conformational change in structure observed between the

proenzyme form of ETA and domain III bound to β-TAD occurred in the loop between residues 457 and 464. In crystals of domain III bound to β-TAD, this loop is moved away from the active site; however, in the proenzyme form of ETA, helix 333–335 of domain II prevents this loop from moving. Presumably, activation of ETA enzymatic activity removes the steric constraints of helix 333–335 in domain II, thus allowing the loop between residues 457 and 464 to move making the extended cleft accessible to NAD^+ (Domenighini et al. 1996; Li et al. 1996).

Domenighini et al. (1991) used computer-based molecular modeling of the NAD^+-binding sites of ETA and diphtheria toxin to identify specific residues that may be involved in catalysis or NAD^+ binding. Seven residues, which are present at equivalent sites in the two toxins, were identified. For ETA, these residues are: His-440, Trp-466, Phe-469, Tyr-470, Tyr-481, Glu-553, and Trp-558. Chemical modification or site-directed mutagenesis of several of these residues has confirmed their role in NAD^+ binding and/or catalytic activity. Mutations at His-440 reduced ADP–ribosyltransferase activity 1000-fold but had no effect on NAD-glycohydrolase activity or NAD^+ binding (Han and Galloway 1995). Han and Galloway (1995) proposed that His-440 is involved in the transfer of the ADP–ribose moiety to elongation factor 2. Glu-553 was initially identified as an active-site residue by photoaffinity labeling (Carroll and Collier 1987). Replacement of Glu-553 with Asp reduced ADP–ribosyltransferase activity 3200-fold and cytotoxicity 400 000-fold (Douglas and Collier 1987, 1990). Using site-directed mutagenesis, Lukac et al. (1988) constructed a mutant toxin with Glu-553 deleted. The mutant toxin was devoid of ADP–ribosyltransferase activity and cytotoxic activities. When administered to mice, 400 lethal doses produced no ill effects. This mutant toxin could block binding of native ETA to LRP and elicited high levels of neutralizing anti-ETA antibodies in mice. Beattie et al. (1996) substituted phenylalanine for the domain-III tryptophan residue Trp-466. ADP–ribosyltransferase and NAD^+-glycohydrolase activities were reduced 20- and threefold, respectively. Beattie et al. (1996) proposed that Trp-466 plays an indirect role in ADP–ribosyltransferase activity by maintaining the structural integrity of the active site. Substitution of Trp-417 and Trp-558 with phenylalanine had minimal effects on ADP–ribosyltransferase and NAD^+-glycohydrolase activities. Mutation of Tyr-481 to Phe resulted in a tenfold reduction in ADP–ribosyltransferase activity and cytotoxicity, but no reduction in NAD-glycohydrolase activity; iodination of this residue abolished ADP–ribosyltransferase activity (Brandhuber et al. 1988; Lukac and Collier 1988). Mutation of Tyr-470 to Phe had no effect on ADP–ribosyltransferase activity, cytotoxicity, or NAD^+-glycohydrolase activity, implying that this residue is not directly involved in catalysis (Lukac and Collier 1988).

An additional residue required for ADP–ribosyltransferase activity (but not involved in NAD^+ binding) was identified by determining the nucleotide sequence of CRM66, an immunologically cross-reactive, ADP–ribosyltransferase-deficient molecule produced by *P. aeruginosa* strain PAO-PRI (Wick

and IGLEWSKI 1988; WOZNIAK et al. 1988). In CRM66, His-426 is replaced with tyrosine. When Tyr-426 of CRM66 was replaced with histidine (a change which renders CRM66 identical to wild-type ETA), cytotoxicity and ADP–ribosyltransferase activity were restored (GALLOWAY et al. 1989). Using an immobilized-elongation-factor-2-binding assay, KESSLER and GALLOWAY (1992) have shown that CRM66 cannot bind to immobilized elongation factor 2, implying that His-426 interacts with elongation factor 2.

In summary, the NAD-binding and catalytic sites of ETA share a conserved three-dimensional structure with other ADP–ribosylating enzymes (DOMENIGHINI and RAPPUOLI 1996). Specifically, these toxins share a common folding of the NAD-binding site, conserved amino acids that are involved in catalysis, and a requirement for a conformational change. In the current model, the NAD-binding and catalytic sites of these toxins are formed by a β-strand followed by a slanted α-helix. Conserved motifs in these toxins include: (1) an aromatic amino acid followed by a histidine (His-440 in ETA) or arginine residue, (2) the motif Glu/Gln (Glu-553 in ETA)–X–Glu, and (3) in ETA and diphtheria toxin, a Tyr–X_{10}–Tyr NAD-binding motif (Tyr-470–Tyr-481 in ETA).

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CHAPTER 5

Diphtheria-Toxin-Based Fusion-Protein Toxins Targeted to the Interleukin-2 Receptor: Unique Probes for Cell Biology and a New Therapeutic Agent for the Treatment of Lymphoma

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A. Introduction

ONTAK (denileukin diftitox) is the trade and generic name for the diphtheria-toxin-based interleukin-2 (IL-2) fusion-protein toxin DAB389IL-2 (WILLIAMS et al. 1987, 1990). The Food and Drug Administration has recently granted approval for the use of ONTAK for the treatment of patients who present with persistent or recurrent cutaneous T-cell lymphoma (CTCL) and whose malignant cells are shown to express the low-affinity p55 chain (CD25) of the IL-2 receptor. Since ONTAK is the first of the rationally designed, targeted fusion-protein toxins to be approved for human use, we shall, in this chapter, review the pre-clinical and clinical development of this biologic and discuss the use of this agent as a novel probe for furthering our understanding of the molecular mechanism by which the catalytic domain of DAB389IL-2 is translocated to the cytosol of target eukaryotic cells.

B. Diphtheria-Toxin-Based Cytokine Fusion Proteins

By the early 1980s, both biochemical and genetic evidence supported the hypothesis that native diphtheria toxin is a protein with at least three domains: an N-terminal, enzymatically active fragment (fragment A), which catalyzes the nicotinamide adenine dinucleotide dependent adenosine diphosphate (ADP)–ribosylation of eukaryotic elongation factor 2 (EF2); a middle portion, which was postulated to facilitate the delivery of fragment A to the eukaryotic cell cytosol; and a C-terminal portion, which serves as the native receptor-binding domain of the toxin. The middle and C-terminal portions of the toxin comprise fragment B (DRAZIN et al. 1971; GILL and PAPPENHEIMER 1971; UCHIDA et al. 1971; BOQUET et al. 1976). In addition, it was clear that, upon binding to its cell-surface receptor [a heparin-binding epidermal growth factor (EGF)-like precursor (NAGLICH et al. 1992)], diphtheria toxin is internalized by receptor-mediated endocytosis. The productive delivery of fragment A to the cytosol requires the toxin to traffic through an acidic compartment (SANDVIG and OLSNES 1980; MOYA et al. 1985). The solution of the X-ray crystal structure of native diphtheria toxin confirmed and provided precise definition of the three-domain structure. Fragment A is composed of the N-terminal cat-

alytic (C) domain, and fragment B is composed of the centrally positioned transmembrane (T) domain and a C-terminal receptor-binding (R) domain (CHOE et al. 1992; BENNETT et al. 1994).

With the advent of methodology to isolate monoclonal antibodies in the mid-1970s, it was envisioned that the catalytic domain (i.e., A chain, or A fragment) of either a microbial or plant toxin could be chemically coupled to a monoclonal antibody, resulting in the formation of cell-specific cytotoxic agents. Furthermore, these agents might serve as prototypes in the development of new human biologics (GILLILAND et al. 1980; VITETTA et al. 1983). In much of this early work, conjugate toxin molecules were assembled by chemically cross-linking (through a disulfide bond) a targeting ligand and either the A-chain of ricin and/or fragment A of diphtheria toxin. An interesting early observation was that conjugates assembled with ricin A chain were highly potent whereas, even at high concentrations, identical conjugates assembled with fragment A of diphtheria toxin were non-toxic (CAWLEY et al. 1980). Since KAGAN et al. (1981) and DONOVAN et al. (1981) demonstrated that the fragment-B portion of diphtheria toxin retained in CRM45 (i.e., a non-toxic mutant of diphtheria toxin lacking the native receptor-binding domain) was capable of inserting into planar lipid bilayers, BACHA et al. (1983) examined the potential role that this portion of diphtheria toxin played in the delivery of fragment A to the cytosol of target cells. In this study, conjugates were assembled with a modified thyrotropin-releasing hormone (TRH) to which both fragment A and CRM45 were separately cross-linked. While both conjugate toxins were found to bind with high affinity to the cell-surface TRH receptor, only CRM45-TRH was found to be toxic [concentration required to inhibit protein synthesis in target cells by 50% (IC50) = 3×10^{-9}M] for rat pituitary cells in culture. Since both conjugate toxin molecules bound to the TRH receptor and were internalized by receptor-mediated endocytosis, these results provided the first demonstration that the N-terminal portion of diphtheria toxin fragment B (i.e., the transmembrane domain) was both essential and sufficient to deliver fragment A across the eukaryotic cell membrane and into the target cell cytosol. Given the variation in specific toxicity found in different preparations of chemically cross-linked TRH–CRM45, we turned to recombinant DNA and protein engineering methods to assemble chimeric targeted toxins at the level of the gene. We reasoned early on that construction of chimeric genes encoding fusion-protein toxins would result in the expression of precisely defined recombinant fusion proteins that could be subjected to detailed structure–function analysis by site-directed mutagenesis.

The underlying rationale for the development of microbial or plant toxin-based polypeptide hormone/growth factor fusion-protein toxins was that substitution of the native receptor-binding domain with a surrogate ligand would result in the formation of a fusion-protein toxin directed toward only those cells that expressed surface receptors for that ligand. Implicit in this rationale is the assumption that, once expressed in recombinant *Escherichia coli*, the fusion-protein toxin would, under appropriate conditions, fold into a biologi-

cally active conformation. Given these assumptions, the fusion-protein toxin should bind to its receptor on the surface of target cells and, once bound, the complex would be internalized into the cell, and the delivery of its catalytic domain to the cytosol would be facilitated. Ironically, the first of the diphtheria-toxin-related fusion-protein toxins were approved by the Recombinant DNA Advisory Committee at biosafety level four, whereas the fusion-protein toxins constructed with the equally potent *Pseudomonas* exotoxin A were approved at biosafety level two.

The genetic construction and properties of the first of the diphtheria-toxin-based fusion-protein toxins was described by MURPHY et al. (1986). These investigators took advantage of an internal SphI restriction endonuclease site located in the C-terminal region of the fragment-B-encoding portion of the *tox* gene. The construct was composed of fragment A and all but the C-terminal 50 amino acids of fragment B (i.e., amino acids 1–486 of native diphtheria toxin, DAB489) genetically fused to a synthetic gene encoding α-melanocyte-stimulating hormone (α-MSH). This initial fusion-protein toxin was expressed in precursor form and secreted into the periplasmic compartment of recombinant *E. coli*. Partially purified DAB486α-MSH was found to be highly cytotoxic for α-MSH-receptor-bearing human malignant melanoma cells in vitro, whereas cell lines that were sensitive to native diphtheria toxin but did not express the α-MSH receptor were found to be markedly resistant.

In these early experiments, it was apparent that DAB486α-MSH was extremely sensitive to proteolytic degradation when purified from recombinant *E. coli*. In order to avoid these difficulties, we next turned our attention to the genetic construction of a protein-fusion toxin in which the 133-amino-acid sequence of human IL-2 was substituted for the native receptor-binding domain. Remarkably, the resulting fusion protein, DAB489IL-2, was found to be expressed in full length and good yield in recombinant *E. coli*. As anticipated, this fusion-protein toxin was found to be highly active against eukaryotic cells that displayed the IL-2 receptor (WILLIAMS et al. 1987). BACHA et al. (1988) demonstrated that the cytotoxic action of DAB486IL-2 was mediated through the high-affinity IL-2 receptor and, once bound to its cell-surface receptor, the fusion-protein toxin was internalized and required passage through an acidic compartment in order to deliver its catalytic domain to the cytosol of target cells. In addition, these investigators demonstrated that, once the catalytic domain of DAB486IL-2 was delivered to the cytosol, it catalyzed the ADP–ribosylation of EF2. Both WATERS et al. (1990) and RE et al. (1996) have shown that the action of the IL-2-receptor-targeted fusion-protein toxins was mediated through the high (α, β, γ-chain)- and intermediate (β, γ-chain)-affinity forms of the IL-2 receptor in both continuous cell lines and peripheral blood monocyte cells (PBMC) in vitro. In these studies, target cells that expressed the high-affinity form of the IL-2 receptor were the most sensitive ($IC50 = 1–10 \times 10^{-12}$ M), followed by cells that expressed the intermediate form of the receptor ($IC50 = 1–10 \times 10^{-10}$ M). In contrast, cells that display the low-affinity (α-, γ-chain) form of the IL-2 receptor were resistant to the action of

these fusion-protein toxins (IC50 > 10^{-7} M; RE et al. 1996). Thus, the mechanism of action of DAB486IL-2 for target cells that expressed the high- or intermediate-affinity receptor for IL-2 was analogous to that of native diphtheria toxin. Most importantly, the highly targeted action of DAB486IL-2 argued for the further development of this agent as an experimental therapeutic for those diseases in which cells that displayed the high-affinity form of the IL-2 receptor were directly involved in pathogenesis.

By constructing a series of internal in-frame-deletion mutations within the structural gene encoding DAB486IL-2, WILLIAMS et al. (1990) genetically mapped the minimum number of diphtheria-toxin-related amino acids in the fusion protein that were necessary for the efficient delivery of the catalytic domain to the cytosol. The in-frame deletion of 97 amino acids from the C-terminal region of diphtheria-toxin-related sequences gave rise to a variant, DAB389IL-2, which was tenfold more potent for cells that expressed the high-affinity IL-2 receptor. In contrast, the further deletion of an additional 18 amino acids gave rise to a variant, DAB371IL-2, which was completely non-toxic. Since DAB371IL-2 bound to the high-affinity IL-2 receptor with essentially the same K_d as DAB389IL-2, it was apparent that amino acids between 371 and 389 of diphtheria toxin played an essential role in the productive delivery of the catalytic domain to the cytosol of target cells. With the solution of the X-ray structure of native diphtheria toxin (CHOE et al. 1992), it became apparent that the region between amino acids 371 and 378 was positioned in transmembrane helix 9, a helix required for the formation of productive channels in the transmembrane delivery of the catalytic domain (VANDERSPEK et al. 1994a). Most importantly, however, was that amino acid 386 was positioned at the end of a random coil linking the transmembrane and receptor-binding domains of the native toxin. Thus, the genetic fusion of human IL-2 sequences to amino acid 389 of diphtheria toxin gave rise to a fusion-protein toxin in which there was almost perfect receptor-binding-domain substitution. As shown in Table 1, a series of diphtheria-toxin-based fusion-protein toxins have been genetically constructed using amino acid 389 of diphtheria toxin as the protein-fusion junction. In the case of DAB389α-MSH, it is of interest to note that the fusion protein is expressed in good yield as a full-length fusion-protein toxin. These results strongly suggest that endoproteolytic degradation of DAB486α-MSH was primarily directed to sites between amino acids 389 and 486 of the native toxin.

While both DAB486IL-2 and DAB389IL-2 were remarkably potent and their action was directed toward target cells bearing the high- and intermediate-affinity forms of the IL-2 receptor in vitro, each of these closely related fusion proteins were found to have unique biochemical characteristics. Fusion proteins are by definition "non-evolved", and problems associated with their expression, refolding into a biologically active conformation, and physical biochemistry often present challenges. For example, in the case of DAB486IL-2, human IL-2 sequences were genetically fused to the middle of the native receptor-binding domain of the toxin. As a consequence, this fusion

Table 1. Diphtheria-toxin-related cytokine/peptide hormone fusion-protein toxins

Toxin	Receptor targeted	IC50	Reference
DAB486α-MSH	α-MSH	n.d.	MURPHY et al. (1986)
DAB389α-MSH	α-MSH	3×10^{-11}	WEN et al. (1991)
DAB486IL-2	IL-2	1×10^{-11}	WILLIAMS et al. (1987)
DAB389IL-2	IL-2	1×10^{-12}	WILLIAMS et al. (1990)
DAB389mIL-3	mIL-3	3×10^{-10}	LIGER et al. (1997)
DAB389mIL-4	mIL-4	2×10^{-10}	LAKKIS et al. (1992)
DAB389IL-6	IL-6	2×10^{-11}	JEAN and MURPHY (1992)
DAB389IL-7	IL-7	1×10^{-10}	SWEENEY et al. (1998)
DAB389IL-15	IL-15	7×10^{-9}	VANDERSPEK et al. (1995)
DAB389EGF	EGF	1×10^{-11}	SHAW et al. (1991)
DAB389CD4	HIV gp120	1×10^{-9}	AULLO et al. (1992)
DAB389sP	sP	5×10^{-12}	FISHER et al. (1996)
DAB389-NT4	p75LNGFR/TrkB	2×10^{-11}	NEGRO and SKAPER (1997)
DAB389GRP	GRP	2×10^{-11}	VANDERSPEK et al. (1997)
DAB389GM-CSF	GM-CSF	1×10^{-11}	BENDEL et al. (1997)
DAB389GM-CSF	GM-CSF	7×10^{-12}	KREITMAN and PASTAN (1997)

EGF, epithelial growth factor; *GM-CSF*, granulocyte–macrophage colony-stimulating factor; *GRP*, gastrin-releasing peptide; *HIV*, human immunodeficiency virus; *IC50*, inhibiting concentration in 50% of subjects; *IL*, interleukin; *LNGFR*, low-affinity nerve growth-factor receptors; *mIL*, murine interleukin; *MSH*, melanocyte-stimulating hormone; *n.d.*, not determined; *sP*, substancep.

protein tends to form aggregates primarily through hydrophobic interactions. In contrast, the fusion of IL-2 sequences in DAB389IL-2 represents an almost perfect domain substitution, and, as a result, the physical biochemistry of this form of the fusion-protein toxin more closely mimics that of an evolved protein (i.e., when refolded from inclusion-body preparations, DAB389IL-2 readily forms biologically active monomers and is stable for extended periods). While these two fusion-protein toxins are closely related and selectively inhibit protein synthesis in IL-2-receptor-bearing target cells, there are important differences between their respective pharmacologic properties in vivo.

C. DAB389IL-2 as a Novel Biologic Probe for Cell Biology

The fusion-protein toxins in general, and DAB389IL-2 in particular, have served as novel probes for the study of interactions between the toxin and its target cell. While the major steps in the intoxication process are known, there is still much to be learned about the precise molecular mechanisms that lead to the delivery of the catalytic domain from the lumen of the endocytic vesicle to the eukaryotic cytosol. Since the binding of DAB389IL-2 to the IL-2 receptor is highly specific, the cytotoxic potency of this fusion-protein toxin has been

used to examine many of the steps in the intoxication process. It is well known that the first step in the intoxication process is the binding of DAB389IL-2 to its cell-surface receptor. VANDERSPEK et al. (1996) used site-directed mutagenesis to examine structure–function relationships in the receptor-binding domain of DAB389IL-2. In this study, mutants were evaluated for their intoxication kinetics, dose–response relationships, and relative ability to bind to the IL-2 receptor and inhibit protein synthesis. Compared with the wild-type fusion-protein toxin, the T439P mutant appeared to have altered binding and signaling. In contrast, the Q514D mutant appeared to have altered kinetics of internalization into the cell.

DAB389IL-2 binds to the high-affinity form of the IL-2 receptor and induces signal transduction, resulting in stimulation of RNA and DNA synthesis (WALZ et al. 1989). Recent results (ZENG and MURPHY, unpublished) suggest that, soon after binding to its receptor, the C domain of DAB389IL-2 becomes denatured and loses its ADP–ribosyltransferase activity. The fusion toxin is then processed by the cellular endoprotease furin, which introduces a "nick" in the α-carbon backbone of the fusion toxin at Arg194 (WILLIAMS et al. 1990; TSUNEOKA et al. 1993). While the transmembrane domain of both native diphtheria toxin and DAB389IL-2 are clearly required for facilitating the delivery of the catalytic domain, the precise roles played by each of the individual α helices within this domain remain obscure. As the fusion toxin is internalized into the early endosomal compartment, the transmembrane domain of the fusion toxin appears to stabilize the toxin on the endosomal membrane by the amphipathic first helical layer (helixes 1–3). As this compartment is acidified, the insertion of the third helix layer of the transmembrane domain, helixes 8 and 9, may stabilize the toxin in the membrane and thereby facilitate the insertion of the second α-helix layer of the transmembrane domain (α helixes 5, 6 and 7). As the nascent channel is forming, the denatured catalytic domain appears to be translocated by a mechanism that is likely to involve the insertion of transmembrane helix 1 into the channel. In this model, transmembrane helix 1 would function to thread the C-terminal end of the catalytic domain through the channel such that the N-terminal end of fragment B and the C-terminal end of the C domain would be delivered through the channel and presented to the cytosol. VANDERSPEK et al. (1994b) demonstrated that maintenance of the hydrophobic surface of transmembrane helix 1 is essential for the efficient delivery of the catalytic domain to the cytosol of target cells. More recently, SENZEL et al. (1998) have shown that the N-terminal portion of the diphtheria-toxin transmembrane domain is translocated through channels formed in planar lipid membranes.

Preliminary studies by ZENG and MURPHY (unpublished) suggest that, once the first few hydrophobic residues of the C-terminal portion of the catalytic domain reach the cytosol, it is possible that cellular chaperonins facilitate the entry process by sequentially binding and refolding the denatured polypeptide into an active conformation as it emerges from the lumen of the early endosome. In the case of the importation of proteins into mitochondria,

it has been shown that chaperonins facilitate membrane translocation, perhaps by the sequential binding and refolding of the denatured protein as it emerges from the inner membrane surface. It is intriguing to postulate that the delivery of the catalytic domain of DAB389IL-2 to the cytosol of target cells employs cellular factors in an analogous fashion and that refolding into an active conformation occurs concomitantly.

D. Pre-Clinical Characterization of DAB486IL-2 and DAB389IL-2

Since both DAB486IL-2 and DAB389IL-2 were selectively active towards cells which displayed the high-affinity form of the IL-2 receptor, a number of in vivo studies were performed to evaluate the potential pharmacologic efficacy of these fusion-protein toxins. It is well known that the de novo expression of the α-chain (CD25, p55) of the IL-2 receptor is a critical and pivotal event in the development of an immune response. KELLEY et al. (1988) evaluated the effects of DAB486IL-2 on the development of delayed-type hypersensitivity (DTH) in a murine model. Mice were immunized with trinitrobenzenesulfonate (Tnbs) and, after 7 days of rest, were challenged by injection of Tnbs into the hind footpad. The treatment regimen consisted of daily intraperitoneal injection of DAB486IL-2 from day zero to day six. Compared with the control groups, treatment was found to be dose dependent and, at 50 μg/day, to abolish a DTH response. Perhaps most importantly, this study demonstrated that Tnbs-immunized animals carried large numbers of CD24-positive CD4+ and CD8+ cells in the draining lymph nodes, whereas immunized animals treated with DAB486IL-2 failed to do so. These results suggest that Tnbs-reactive lymphocytes expressing the high-affinity IL-2 receptor were selectively eliminated during the treatment course.

BACHA et al. (1992) determined the effect of DAB486IL-2 on the development of adjuvant arthritis in the rat. Rats treated during the induction phase of arthritis were found to have delayed onset of symptoms and a marked reduction of inflammation. These results also suggested that the administration of DAB486IL-2 during the induction phase of the disease was able to largely eliminate the emergence of an immune response in vivo. Both of these early studies strongly suggested that DAB486IL-2 is capable of selective targeting and elimination of activated lymphocytes, resulting in modulation of the immune response.

KIRKMAN et al. (1989) evaluated DAB486IL-2 as an immunosuppressive agent in a murine vascularized, heterotopic heart-transplant model. In this system, hearts from B10.BR mice were transplanted into C57B1/10 recipients. Following transplantation, mice were treated with 1 μg/day DAB486IL-2 for 10 days. Compared with CRM45-treated controls, animals that received the fusion-protein toxin maintained their heart transplants for extended periods of time.

The analysis of atherosclerotic lesions in both human and experimental animals has revealed the presence of activated lymphocytes and monocytes in the pathogenesis of post-angioplasty restenosis. As a result of these observations, MILLER et al. (1996) examined the effects of DAB486IL-2 administration following balloon angioplasty in rabbits. In this study, 11 rabbits were treated with the fusion-protein toxin at a level of 100 μg/kg/day for 10 days post-angioplasty. After 6 weeks, the treated rabbits showed no change in the minimum luminal diameter at the angioplasty site whereas, in the placebo group (11 animals), the cross-sectional area at the angioplasty site was reduced by $34 \pm 21\%$ ($P = 0.01$).

RAMADAN et al. (1996) examined the effect of the second-generation form of the IL-2-receptor-targeted fusion toxin, DAB389IL-2, in a murine model of schistosomiasis. In the case of schistosomiasis, clinical disease is caused by an immunopathologic response to parasite ova that are deposited in the tissues of the host. DAB389IL-2 treatment was found to suppress the development of granulomas and collagen deposition in the livers of infected animals.

In the studies summarized above, both DAB486IL-2 and DAB389IL-2 were found to be potent immunosuppressive agents in several animal models of disease and in a murine model of cardiac transplantation in vivo. These studies underscore the potential utility of these IL-2-receptor-targeted fusion-protein toxins in the treatment of those human diseases in which activated lymphocytes and monocytes play either a direct or indirect role in pathogenesis. It is likely that several attributes of these fusion-protein toxins contribute to their in vivo efficacy: (1) their cytotoxic potency, (2) their selectivity for activated lymphocytes that display the high-affinity form of the IL-2 receptor, (3) their relatively low K_d for binding to the IL-2 receptor, and (4) their relatively short half-life in circulation (BACHA et al. 1990). In concert, these factors appear to allow for the selective elimination of activated lymphocytes present at the time of fusion-protein-toxin administration. Moreover, since the half-life in circulation is relatively short, prolonged non-specific, cytotoxic effects would not be anticipated. However, in contradiction to the notion that "if a small dose is good, then a large dose will be better", the extraordinary potency of either DAB486IL-2 or DAB389IL-2 targeted fusion toxins suggests that the administration of relatively low but therapeutically effective doses over a longer period of time will likely be most efficacious. Indeed, JACOBSON et al. (1996) have found that administration of daily low doses of recombinant native IL-2 to human immunodeficiency virus-positive individuals over an extended period enhanced immune function without the extreme toxicity normally associated with the administrate of this cytokine.

E. Clinical Evaluation of DAB486IL-2 and DAB389IL-2

In general, there is an urgent medical need for the development of new agents for the treatment of both malignant and autoimmune disease. The progression

of disease in both settings often results in the emergence of resistance to chemotherapy and treatment failure. The diphtheria-toxin-based cytokine fusion proteins offer several desirable properties that one would like to employ in the development of new therapeutics. For example, the fusion-protein toxins are extremely potent and capable of inhibiting protein synthesis and eliminating target cells at picomolar concentrations. In contrast, cells that are devoid of the targeted receptor are resistant and require 10^4 to 10^5 fold higher concentrations of the fusion toxins to inhibit cellular protein synthesis. In addition, EF2 represents a new and powerful target in therapeutic development. It is well known that EF2 plays an essential role in protein synthesis by catalyzing the movement of the ribosome one codon down on the messenger RNA strand. In this process, EF2 dissociates from the ribosome, binds guanosine triphosphate (GTP), and then returns to the complex. The hydrolysis of GTP then provides the energy for ribosomal translocation. In the intoxication process, once EF2 has been modified by ADP–ribosylation, it can no longer hydrolyze GTP and, as a result, protein synthesis becomes arrested. Since EF2 can only serve as a substrate in the ADP–ribosylation reaction when it cycles off the ribosome, the higher the rate of protein synthesis in a given cell, the more sensitive that cell will be toward an ADP–ribosylating toxin.

The expression of the IL-2 receptor has been reported on a number of human hematologic malignancies, including Hodgkin's disease, low- and intermediate-grade non-Hodgkin's lymphoma, CTCL, human T-lymphotropic-virus-1-associated adult T-cell leukemia/lymphoma, and chronic lymphocytic leukemia (Uchiyama et al. 1985; Waldmann 1990). In addition, the high-affinity receptor for IL-2 is a common and obligatory event in the development of an immune response. The expression of this receptor on auto-aggressive T cells marks an early common event in the pathogenesis of essentially all autoimmune diseases. Taken in aggregate, human autoimmune disease is the most prevalent human disease. Rheumatoid arthritis, psoriasis, autoimmune type-1 onset diabetes, multiple sclerosis, and numerous other autoimmune disorders combine to affect a relatively large percent of the world population. Clearly, the presentation of autoimmune disease ranges from mild to severe and from manageable to refractory. While most autoimmune diseases are generally not life threatening, patients who present with severe refractory illness are in need of new approaches for the treatment of the underlying basis of their disease rather than management of their symptoms. Given the results of preclinical studies of DAB486IL-2 and DAB389IL-2 in a variety of animal models, a series of phase-I/II studies have been conducted in patients with refractory rheumatoid arthritis, psoriasis, and type-1 onset diabetes.

I. Rheumatoid Arthritis

In a phase-I/II protocol, Sewell et al. (1993) treated with DAB486IL-2 19 patients who presented with methotrexate-resistant rheumatoid arthritis. The

fusion toxin was administered intravenously at one of three dose levels daily for 5 days or 7 days. Arthritis response was assessed at 28 days post-treatment. In this study, 9 of 19 patients treated with the high or intermediate dose level had a substantial (>50%) or meaningful (>25%) improvement in their disease. In this patient population, clinical benefit was found to be rapid, and full effects of fusion-protein-toxin administration were noted by 14 days after treatment. The most frequent adverse effects were transient elevations of hepatic transaminases and fever.

This initial clinical trial was followed by a phase-II, double-blind, placebo-controlled study in which 45 rheumatoid-arthritis patients were enrolled (MORELAND et al. 1995). In the double-blinded phase of this study, 4 of 22 treated patients (18%) met the criterion for a clinical response; none of the placebo group met this criterion. In the open-label phase of the study, 11 of 36 patients (31%) and 11 of 33 patients (33%) achieved a clinical response following two and three courses of treatment, respectively.

II. Psoriasis

While the underlying etiology of psoriasis remains unknown, this autoimmune disease is characterized by a hyperplasia of epidermal keratinocytes and a marked infiltration of both CD4+ and CD8+ lymphocytes into psoriatic lesions in the skin. Psoriasis is an extraordinarily common autoimmune disease, affecting approximately 2% of the U.S. population. The disease ranges from mild to a severely disfiguring and debilitating form. Since immunomodulating agents (cyclosporin, FK506, monoclonal anti-CD4+ antibodies) had been shown to induce clinical improvement in patients with severe disease, GOTTLIEB et al. (1995) evaluated DAB389IL-2 in the treatment of patients with a history of long-standing extensive psoriasis. In this refractory population, previously failed therapies included topicals, psoralen followed by ultraviolet-A irradiation (PUVA), cyclosporin, and methotrexate. Following either a 2-week washout period for topical agents or a 4-week period for cyclosporin and PUVA, patients were treated with either 100 kU/kg (2 μg/kg) or 200 kU/kg (4 μg/kg) DAB389IL-2 administered intravenously daily for 5 days. Patients were monitored for 23 days and then treated with a second 5-day regimen. At the end of the second 23-day monitoring period, four patients showed marked clinical improvement, four patients showed moderate improvement, and two patients had only minimal improvement.

Importantly, this study was the first to show that clinical improvement correlated with a marked reduction of psoriatic-lesion-infiltrating T cells (both CD4+ and CD8+ subsets). Given that DAB389IL-2 action is specifically directed towards cells that express the high-affinity form of the IL-2 receptor and that human keratinocytes in culture are resistant to the fusion-protein toxin, this study also provided the first evidence suggesting that psoriasis is an autoimmune disease in which pathology is driven by auto-aggressive T cells rather than by keratinocyte proliferation.

III. Non-Hodgkin's Lymphoma

A series of phase-I/II clinical trials were conducted with DAB486IL-2 and DAB389IL-2 to establish their relative safety, pharmacokinetics, and potential efficacy in the treatment of non-Hodgkin's lymphoma. The first studies were conducted with DAB486 IL-2 in order to establish a "proof-of-principle" for the biological efficacy of IL-2-receptor-targeted cytotoxic therapy in lymphoma (LeMaistre et al. 1992, 1993; Schwartz et al. 1992; Hesketh et al. 1993; Foss and Kuzel 1995; Foss et al. 1998). Patients enrolled in these studies presented with refractory disease and had failed at least two prior chemotherapy treatment regimens. In these initial studies, the presence of IL-2 receptors on tumor biopsy was not a prerequisite for enrollment. Single and multiple doses of the fusion-protein toxin were administered by intravenous injection as a bolus or by 90-min infusions. Dose escalations were performed on three patient cohorts with a starting dose of 700 ng/kg/day, which was gradually increased to 400 μg/kg/day.

Adverse effects associated with the intravenous administration of DAB486IL-2 were transient and included fever, malaise, hypersensitivity, nausea/vomiting, and increased levels of serum hepatic transaminases. The maximum tolerated dose was determined to be 400 μg/kg/day; above this level, renal insufficiency occurred. Time-course studies indicated that this fusion-protein toxin cleared from the serum with a $t_{1/2}$ of 11 min in dose ranges of 200–400 μg/kg (LeMaistre et al. 1993). Increased levels of soluble IL-2 receptor were detected in the serum of many patients but had no effect on either clearance rates or potential efficacy. Furthermore, antibodies to IL-2 developed in 50% of the patients during the course of the study but seemed to have no effect on anti-tumor response. Clinical responses occurred in 8% (4/51) of the patients with low- and intermediate-grade non-Hodgkin's lymphoma, 7% (1/14) of the patients with Hodgkin's disease, and 17% (6/36) of patients with CTCL. One patient with tumor-stage CTCL had a complete remission and has remained disease-free for over 5 years (Hesketh et al. 1993). As anticipated from in vitro studies, it was notable that all patients who responded to DAB486IL-2 therapy had demonstrable IL-2-receptor expression, as shown by immunoreactivity with anti-Tac (CD25) antibody.

These initial human clinical trials with DAB486IL-2 clearly demonstrated that treatment of diverse groups of patients who presented with refractory non-Hodgkin's lymphoma had the potential to offer meaningful results. Thus, these early studies established the "proof of principle" that a fusion-protein toxin directed towards the IL-2 receptor could be administered with a degree of safety and tolerability that warranted further study. As described above, the second-generation form of the fusion toxin, DAB389IL-2, bound to the IL-2 receptor 5-fold more avidly and was at least tenfold more potent in the selective elimination of high-affinity-receptor-bearing cells. Given that this fusion protein had more desirable biophysical properties and was easily purified to clinical grade, all additional human clinical trials were conducted with

DAB389IL-2. Moreover, the need to focus on a single disease entity for potential regulatory approval was dictated, and further clinical trials with DAB389 IL-2 were limited to patients with CTCL, non-Hodgkin's lymphoma, and Hodgkin's disease. The detailed analysis of results from earlier studies suggested that those patients who presented with tumors that expressed the IL-2 receptor might have an increased likelihood of responding; therefore, only those patients with demonstrable IL-2 receptors were enrolled.

As reported by LeMaistre et al. (1998), this group of refractory patients had experienced a mean of five previous therapies and included 25 patients who had received bone-marrow transplants. A cohort dose-escalation trial was once again employed, and patients received doses ranging from 3 μg/kg/day to 31 μg/kg/day for 5 days. Treatment cycles were repeated every 3 weeks. Side effects included fever/chills, nausea/vomiting, malaise, and reversible elevation of serum transaminases. Eight of the patients with CTCL experienced hypoalbuminemia, hypotension, and edema, which were considered to be symptoms of a mild vascular leak syndrome. Dose-limiting toxicity was determined to be 31 μg/kg/day, based on malaise. All observed toxicities were reversible and were not cumulative. Thirty-nine (53%) patients discontinued the study because of disease progression, and 12 (16%) stopped treatment because of toxicity. Of the 73 patients who began the treatments, 52 completed two courses, 39 completed three courses, and 18 completed all six cycles. As in the earlier study, pre-existing antibodies and antibodies developed after treatment with DAB389 IL-2 did not appear to interfere with the clinical response or contribute to adverse reactions. Clinical responses were observed in 16 of the 73 patients (22%), with effects occurring in 13 out of 35 patients (37%) with CTCL and 3 of the 17 (18%) non-Hodgkin's-lymphoma patients. DAB389 IL-2 did not elicit responses in Hodgkin's-disease patients. The median response was found to occur after two courses of treatment, and the median duration of response was 10 months. Since four of five complete responses were found in those patients who presented with CTCL, phase-III studies of DAB389IL-2 were initiated in this patient population.

IV. Cutaneous T-Cell Lymphoma

As a clinical entity, CTCL describes a series of low-grade non-Hodgkin's lymphomas in which malignant T cells invade the skin (Broder and Bunn 1980). This malignancy is also known as either mycosis fungoides or, in its erythrodermic leukemic variant, Sezary syndrome. While mycosis fungoides is a disease of the skin in its early stages, in later stages, lymph nodes, spleen, liver, and other organs may also be involved. Skin lesions associated with this disease usually progress through patch, plaque, and tumor phases. Patch lesions are flat, scaly, erythematous macules which may itch, whereas plaques are generally raised, red to purple in color, and may be thick and scaly and usually intensely itchy. Reddened nodular tumors may arise from plaque lesions and tend to predominate on the face and other areas of the body. With

time, tumorous lesions usually become ulcerated and prone to secondary infection. Tumors may also spread to regional lymph nodes and visceral organs. In the case of Sezary syndrome, atypical lymphocytes are found in peripheral blood.

Patients who present with CTCL become symptomatic even in early stages of their disease. Both the breakdown of the normal skin barrier and depression of their cell-mediated immune response is compromising, and this patient population is predisposed toward infection (Axelrod et al. 1992). While the 8-year to 10-year overall survival rate for these patients is similar to those with other non-Hodgkin's lymphomas, once disease has progressed to lymph-node or organ-system involvement, the median survival is less than 3 years. While there are many therapeutic regimens that have shown efficacy in early-stage disease, patients with advanced disease are refractory, and complete clinical responses are rare. Once disease progresses beyond involvement of more than 10% of the total body surface area, spontaneous remission from disease does not occur, and the disease is usually fatal.

Given the promising results from the open-label phase-II clinical trials with DAB389IL-2, a phase-III clinical study was designed to test rigorously the potential efficacy of treating CTCL patients with this fusion-protein toxin. This trial was designed to include two randomized, double-blinded studies in mutually exclusive patient populations. The first arm of the trial evaluated the intravenous administration of DAB389IL-2 for up to eight courses of therapy at either 9 μg/kg/day or 18 μg/kg/day in patients with advanced refractory disease, whereas the second arm of the trial evaluated DAB389IL-2 in patients with less advanced disease at 9 μg/kg/day and 18 μg/kg/day and included a placebo control group. In the second arm of the study, patients whose disease progressed were un-blinded and, if they had received placebo, they were allowed to enroll in an open-label study in which they received the fusion-protein toxin at 18 μg/kg/day.

Upon completion of the first arm of the phase-III trial, the overall response rate in patients who met the inclusion criteria demonstrated that 30% of the patients had a 50% or greater reduction in their tumor burden for at least 6 weeks following treatment with DAB389IL-2. Ten percent of the patients who were evaluable had either a complete or complete clinical response (i.e., complete responders were histologically free of disease). While there was a trend toward a higher response rate in patients treated at the 18-μg/kg/day level (36%) compared with those treated at 9 μg/kg/day (23%), the total number of patients in each group is too small to allow statistical separation of the two groups.

In this study, the most common adverse events experienced by this patient population was chills/fever, malaise, and nausea/vomiting. Less frequent adverse events were hypotension, edema, rash, and a capillary leak syndrome. As discussed above, CTCL patients suffer a substantial and often disfiguring disability during the course of their illness. Since the analysis of the first arm of the phase-III clinical trail demonstrated that DAB389IL-2 therapy has the

potential to offer substantial reduction in tumor burden and relief from constitutional symptoms in a large percentage of patients who are otherwise refractory, the Food and Drug Administration was petitioned to consider approval of this fusion-protein toxin for patients with refractory CTCL. As noted above, ONTAK (DAB389IL-2) was approved for the treatment of persistent or recurrent CTCL in patients whose malignant cells are shown to express at least the CD25 component of the IL-2 receptor.

While much remains to be done, the fusion-protein toxins directed towards the IL-2 receptor have proven to be interesting probes for the analysis of toxin/cell interactions leading to the intoxication of target cells. Moreover, DAB389IL-2 (ONTAK) has proven to be an effective new biologic agent for the treatment of refractory cutaneous T-cell lymphoma. As this was the first of the fusion-protein toxins to be approved for human use, future development of additional fusion-protein toxins seems promising.

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CHAPTER 6

Structure and Function of Cholera Toxin and Related Enterotoxins

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A. Introduction

Vibrio cholerae and enterotoxigenic *Escherichia coli* (ETEC) both cause diarrheal disease and represent a major health problem, especially among children in developing countries. Cholera is one of the great epidemic diseases; seven pandemics have been recorded in history, the last three of which are known to be due to *V. cholerae* serogroup O1. In 1992, a new cholera epidemic emerged, caused by the new serotype *V. cholerae* O139 Bengal (ALBERT 1994). The new epidemic quickly spread through India and Bangladesh.

Even today, a century after the discovery of the comma-shaped cholera bacillus by KOCH (1884), the number of victims of these diarrheal diseases is still enormous. Annually, cholera causes more than 150,000 deaths, while ETEC diarrhea is responsible for one million deaths (HOLMGREN and SVENNERHOLM 1992). Common travelers' diarrhea is a mild form of the same disease, caused by infection with ETEC. The treatment for diarrheal disease is to replace the lost fluids and electrolytes by re-hydration, either intravenously or orally (RABBANI 1996).

The protein responsible for the onset of diarrhea caused by *V. cholerae* is cholera toxin (CT; DE 1959) while, for a subset of enterotoxigenic *E. coli*, the homologous protein is heat-labile enterotoxin (LT-I; SMITH and HALLS 1967). The two toxins are very similar in structure and are 80% sequence identical for both their A and B polypeptide chains. Most experimental data on CT, which was isolated and characterized first (FINKELSTEIN and LOSPALLUTO 1969), are thus also applicable to LT-I and vice versa. The CT family also includes two more recently discovered heat-labile enterotoxins, LT-IIa and LT-IIb, which are secreted by certain *E. coli* strains (GREEN et al. 1983; GUTH et al. 1986a). The latter two toxins are not (yet) associated with any known diarrheal disease in humans or in animals.

Structural studies on these toxins serve not only as tools in the fundamental investigation of their interesting modes of action but also as aids in the eventual conquest of their respective diseases. Structure-based drug-design efforts (HOL 1986) benefit from the three-dimensional details of the structural basis of toxin assembly, receptor binding, and catalytic activity. The fruits of these investigations may one day lead to one or more small-molecule

prophylactic drugs that block the toxin at critical points in its mode of action. Inducing immunity against these diarrheal diseases would be even better, of course, and structural studies on these toxins may also aid in the development of a safe and potent vaccine. The battle against cholera may be complicated by the recent finding that key pathogenic proteins, including the toxin itself, are encoded by a filamentous bacteriophage, CTXΦ, and can easily be transferred from pathogenic *V. cholerae* strains to other, formerly non-pathogenic strains (WALDOR and MEKALANOS 1996).

Several review articles have been published covering the exciting field of research on LT-I and CT structure–function relationships from its early beginnings in the 1960s until the early 1990s (HOLMGREN 1981; SIXMA 1992; SPANGLER 1992; HOL et al. 1995; and several books devoted to meetings covering bacterial toxins). We concentrate here on more recent work.

The toxins LT-I and CT are heterohexameric proteins comprised of one A subunit and a pentamer of identical B subunits. The A subunit is responsible for the catalytic activity of the toxin, while the B pentamer directs the A subunit to the target intestinal epithelial cells by binding to ganglioside G_{M1}. After membrane translocation, the A subunit catalyzes the adenosine diphosphate (ADP)–ribosylation of $G_{s\alpha}$ present in the cytoplasm, causing this G protein to lose its guanosine triphosphate (GTP)ase activity, thereby leaving it permanently in the activated, GTP-bound state. This activated $G_{s\alpha}$ continuously stimulates adenylate cyclase, which leads to an increase in intracellular cyclic adenosine monophosphate (cAMP). This, in turn, causes fluid and electrolyte secretion, resulting in diarrhea and, if untreated, death by dehydration, especially among children and the elderly. In order for the A subunit to become catalytically active, it must be proteolytically cleaved near residue 193, resulting in the A1 and A2 cleavage products. After this "nick", the A1 subunit, which bears the catalytic activity, and the A2 linker are still covalently coupled by means of a disulfide bond between Cys187 and Cys199, which must be reduced to obtain full activity (SPANGLER 1992; Chap. 1).

B. Three-Dimensional Structures of Holotoxins

The crystal structures of the AB_5 LT-I and CT holotoxins were determined in the early 1990s (SIXMA et al. 1991, 1993; ZHANG et al 1995), and the structure of the B pentamer of cholera toxin was elucidated, first at medium resolution (MERRITT et al. 1994b) and recently at very high resolution (MERRITT et al 1998). Both toxins are very similar in three-dimensional structure (as expected, since the two proteins are 80% identical in sequence). The five B subunits, of 104 amino acid residues each, form a large ring with a diameter of ~60 Å and a thickness of ~40 Å (Fig. 1). The ring encircles a central pore about 30 Å long with a diameter ranging from 11 Å to 15 Å. The interior surface of the pore is highly charged. Through this pore extends the C-terminal tail of the A subunit, the A2 domain, which serves to link the B pen-

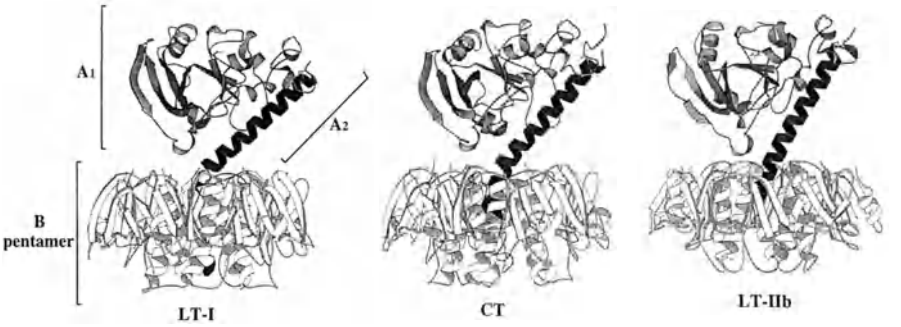

Fig. 1. Secondary-structure representations of the AB5 holotoxins heat-labile enterotoxin (LT)-1 (SIXMA et al. 1991) cholera toxin (ZHANG et al. 1995b), and LT-IIb (VAN DEN AKKER et al. 1996b). The catalytic domain of the A subunit, highly homologous in all three toxins, is in each case shown in *light gray*. The A2 domain of the A subunit, which tethers the remainder of the A subunit to the B pentamer, is shown in *dark gray*. The B pentamer itself is shown in *white*. Each toxin is oriented such that the long helix of the A2 domain lies in the plane of the figure while the fivefold axis through the central pore of the B pentamer is vertical

tamer to the catalytic A1 domain. Many of the interactions between the pore and the A2 domain are water mediated (SIXMA et al. 1993). This most unusual mode of association allows considerable flexibility in the relative orientations of the A1 catalytic domain and the B pentamer (SIXMA et al. 1992a). The one notable difference between the holotoxin structures of LT-I and CT is in the conformation of the A2 domain. The helical portion of A2 in the LT-I structure ends as it enters the B-pentamer pore, and the remainder of the A2 domain descends through the pore in a well-defined extended conformation to form a small helix at the other end of the pore (SIXMA et al. 1991, 1992b, 1993). However, in CT, the A2 domain forms a significantly longer helix and does not seem to be well ordered inside the pore (ZHANG et al. 1995).

The A subunit of 240 residues is a triangular-wedge shape in profile, with the A2 helix lying along one side. As described later, it is structurally homologous to the catalytic domains of other ADP–ribosylating toxins. The A1 domain (Fig. 1) can be divided into three substructures (ZHANG et al. 1995). Subdomain A1-1, comprising residues 1–132, forms the framework of the catalytic domain. Subdomain A1-2, comprising residues 133–161, forms an extended linker from one end of the A1-1 subdomain to near the A1-1/A2 interface. The C-terminal A1-3 subdomain, formed by residues 162–199, is a small, separate, irregular domain around the activation/cleavage site containing the Cys187–Cys199 disulfide bond and a stretch of hydrophobic residues (ZHANG et al. 1995).

The B subunits of LT-I and CT adopt the oligosaccharide- and oligonucleotide-binding fold, called the O/B fold, first observed in staphylococcal nuclease (MURZIN 1993). This motif is conserved in other AB_5-toxin family members, such as verotoxin (STEIN et al. 1992), shiga toxin (FRASER et al. 1994),

and pertussis toxin (PT; STEIN et al. 1994a) and also is found in several other proteins, such as staphylococcal enterotoxin B (SWAMINATHAN et al. 1992), toxic shock syndrome toxin 1 (ACHARYA et al. 1994), and the active domain of the tissue inhibitor of metalloproteinase 2 (WILLIAMSON et al. 1994). The B pentamer of LT-I and CT contains five identical receptor-binding sites, located on the surface furthest from the bulk of the A subunit (SIXMA et al. 1992). The ring-like arrangement of the five B subunits, as first seen for LT by SIXMA et al. (1991), was later also encountered in other AB_5 toxins, including verotoxin (STEIN et al. 1992), shiga toxin (FRASER et al. 1994), CT (ZHANG et al 1998b), LT-II (VAN DEN AKKER et al. 1997) and, with interesting variations, PT (STEIN et al. 1994a).

C. Toxin Assembly and Secretion

The A and B subunits of both LT-I and CT are synthesized intracellularly, each preceded by an N-terminal leader peptide. The leader peptide is cleaved off once the subunits are translocated across the bacterial cytoplasmic membrane. Assembly of the A and B subunits takes place inside the periplasm (HIRST et al. 1984; HOFSTRA and WITHOLT 1984). The assembly of the AB_5 holotoxin involves AB_3 and AB_4 assembly intermediates and cannot occur from preformed B pentamers (HARDY et al. 1988). The assembly of B subunits into pentamers is enhanced by the presence of the A subunit. Residues 226–236 of the A subunit have been shown to be important for this enhancement (STREATFIELD et al. 1992). Arginine residues near position 146 in the A subunit have also been found to be important for holotoxin assembly, although each individual arginine can be mutated to glycine without any noticeable effect (OKAMOTO et al. 1995). One of the mutated arginines is at the A/B_5 interface (SIXMA et al. 1993).

The C-terminal amino acids of the B subunit have been shown to be essential for the formation and stabilization of B-subunit assembly intermediates but do not contribute to the stability of the final pentameric state of the B subunits (SANDKVIST and BAGDASARIAN 1993). The N-terminal ten residues of the B subunits are ordered only in completely assembled B pentamers and can be recognized by the antibody LDS47 only in assembly intermediates (AMIN et al. 1995). The disulfide bond Cys9–Cys86 in the B subunits in CT is essential for the stability of the B pentamer (JOBLING and HOLMES 1991). In the completely assembled CT holotoxin, the stability of the A subunit and the B pentamer are significantly different. The A subunit denatures irreversibly at 51°C, while the B pentamer is stable at temperatures up to 74°C (GOINS and FREIRE 1988).

V. cholerae contains a specific endoproteinase that nicks the A subunit between domains A1 and A2 to partially activate the toxin prior to secretion (FINKELSTEIN et al. 1983). LT-I is not cleaved prior to secretion from *E. coli*. Crystallographic analysis of partially activated LT-I (MERRITT et al. 1994b) and

naturally cleaved CT (ZHANG et al. 1995) indicate that proteolytic cleavage does not cause any significant conformational changes with respect to the uncleaved wild-type LT-I structure, since the cleavage loop is very mobile in both CT and LT-I.

V. cholerae contains machinery to actively secrete CT, while *E. coli* depends on cell lysis to release LT-I into the gut. The secretion apparatus of *V. cholerae* is selective, as the whole periplasmic content is not released. In *V. cholerae*, the epsE gene and 11 additional genes are known to be involved in CT secretion (OVERBYE et al. 1993). The extracellular transport signal is located on the B subunits (HIRST and HOLMGREN 1987) and has recently been postulated to be near Glu11 (CONNELL et al. 1995). However, *V. cholerae* can secrete not only LT-I B pentamers (HIRST et al. 1984) but also the B pentamers of LT-IIa and LT-IIb, which both have very little sequence similarity with CT (CONNELL et al. 1995). This suggests that the extracellular transport signal in *V. cholerae* is most likely a conformation-dependent motif (CONNELL et al. 1995).

I. Design of Assembly Antagonists

The inability of the AB_5 holotoxin to assemble from pre-formed B pentamers suggests that it may be possible to prevent the formation of holotoxin by interfering with the normal periplasmic assembly process. Following the suggestion of VAN DEN AKKER et al. (1997) that the association of the A subunit with nascent B pentamers may be guided by a ring of hydrophobic residues at the mouth of the central pore, HOVEY et al. (1999) found a small molecule that binds to this region of the B pentamer (Fig. 2). If such a compound can be developed to the point where it competes effectively with the A subunit during holotoxin assembly in the periplasm, this would constitute a novel approach to toxin inhibition. The toxin-secretion apparatus is also a hypothetical target for drug design, at least in the case of CT, but information about the proteins involved is insufficient to enable pursuit of this goal.

D. Cell-Surface-Receptor Recognition

Although receptor binding by the AB_5 toxin family is always mediated by the B subunits, the specific receptor-binding site on the toxin is not structurally conserved across the family (SAUKKONEN et al 1992; LING et al. 1998). LT-I and CT, however, exhibit a virtually identical receptor-binding site that specifically recognizes the cell-surface pentasaccharide belonging to ganglioside GM1. LT-I, but not CT, has also been shown to bind weakly to cell-surface glycoproteins in addition to binding GM1 (HOLMGREN 1994). No specific details of this alternative binding interaction have been reported.

One GM1-binding site is present on each monomer of the B pentamer (SIXMA et al 1991; 1992; MERRITT et al. 1994; HOL et al. 1995); hence, the toxin

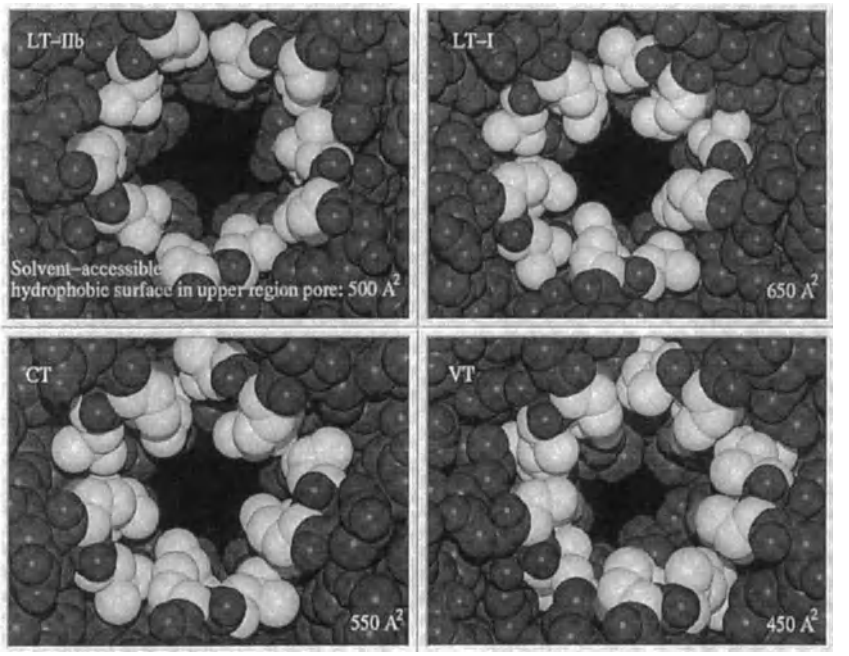

Fig. 2. The hydrophobic ring at the entrance of the B_5 pores of heat-labile enterotoxin (LT)-I, cholera toxin (CT), LT-IIb and verotoxin (VT)

can bind to five molecules of GM1 on the target cell. The 61-kDa complex of the CT B pentamer bound to the complete GM1 oligosaccharide has been studied crystallographically at the unusually high resolution of 1.25 Å (Merritt et al 1998). Structural details of the toxin–receptor binding interaction have also been studied through a series of structures containing receptor fragments bound to the LT-I holotoxin or B pentamer (Sixma et al. 1992; Merritt et al. 1994a; Van den Akker et al. 1996a).

The picture which has emerged is that of a relatively rigid receptor (the terminal four sugars of the GM1 oligosaccharide) bound by a relatively flexible binding site on the toxin. In the absence of bound sugars, residues 50–60 of the toxin are only poorly ordered. Upon binding galactose (a minimal receptor fragment) or larger receptor analogues, however, these residues become well ordered and contribute to the toxin–receptor interaction surface. The role of individual toxin residues in receptor recognition has been probed both genetically and structurally (Jobling and Holmes 1991; Merritt et al. 1995, 1997a). The major binding interaction involves the terminal galactose residue of GM1, which is inserted deeply into a cavity of the toxin surface. The hydrophobic face of the galactose sugar ring stacks against the side chain of Trp88, while the galactose hydroxyl groups form extensive hydrogen-bonding

networks with toxin residues Gly33, Glu51, Gln56, Asn90, and Lys91. Additional binding interactions are contributed by the *N*-acetylgalactosamine and sialic-acid sugar residues of GM1. The rigidity of the GM1 oligosaccharide is maintained by additional intra-saccharide hydrogen-bonding and steric interactions among these same three sugar residues.

I. Design of Receptor Antagonists

The toxin's receptor-binding site is a particularly attractive target for drug design, because it is accessible to orally administered compounds in the gut. That is, in order to block toxin binding, an inhibitor need not enter either the target host cell or the bacterium itself. The extensive structural information on the toxin–receptor binding interaction has allowed the identification and design of small molecules that constitute lead compounds in the development of receptor-blocking inhibitors. The first generation of such compounds is based on the addition of new moieties to galactose (Fig. 3; Merritt et al. 1997b; Minke et al. 1999), but more diverse chemistry may emerge during subsequent rounds of the iterative process of structure-based inhibitor design.

E. Toxin Internalization

Internalization takes place via non-coated vesicles (Tran et al. 1987) generated by caveoli (Parton et al. 1994; Orlandi and Fishman 1998; Wolf et al. 1998) or possibly by the endosomal pathway (Sofer and Futerman 1995). Ganglioside GM1 may act as a transducer, allowing a pH-dependent conformational change in the bound toxin to cause a change in the local membrane structure (McCann et al. 1998). The process of toxin entry into the cell has been traced using fluorescent confocal microscopy (Bastiaens et al. 1996; Majoul et al. 1996). These authors concluded that: (1) after binding GM1, the holotoxin is internalized into vesicles; (2) the holotoxin is transported to a Golgi compartment; (3) the A subunit dissociates from the B subunits in the perinuclear region of the Golgi cisternae; (4) the A subunit is directed, via retrograde transport, from the Golgi to the endoplasmic reticulum, while the B subunits remain in the Golgi; and (5) either the A subunit or the A1 domain alone is translocated into the cytosol. The four C-terminal residues of the A subunit (RDEL in LT-I; KDEL in CT) constitute a eukaryotic signal for intracellular trafficking that normally directs the retention of proteins in the endoplasmic reticulum (Pelham 1989). This is consistent with the observed retrograde transport of the toxin from Golgi to endoplasmic reticulum, but there is contradictory experimental evidence for the involvement of this tetrapeptide signal in toxicity (Cieplak et al. 1995; Lencer et al. 1995b; Sandvig et al. 1996). Additional discussion of toxin internalization may be found in Chap. 1.

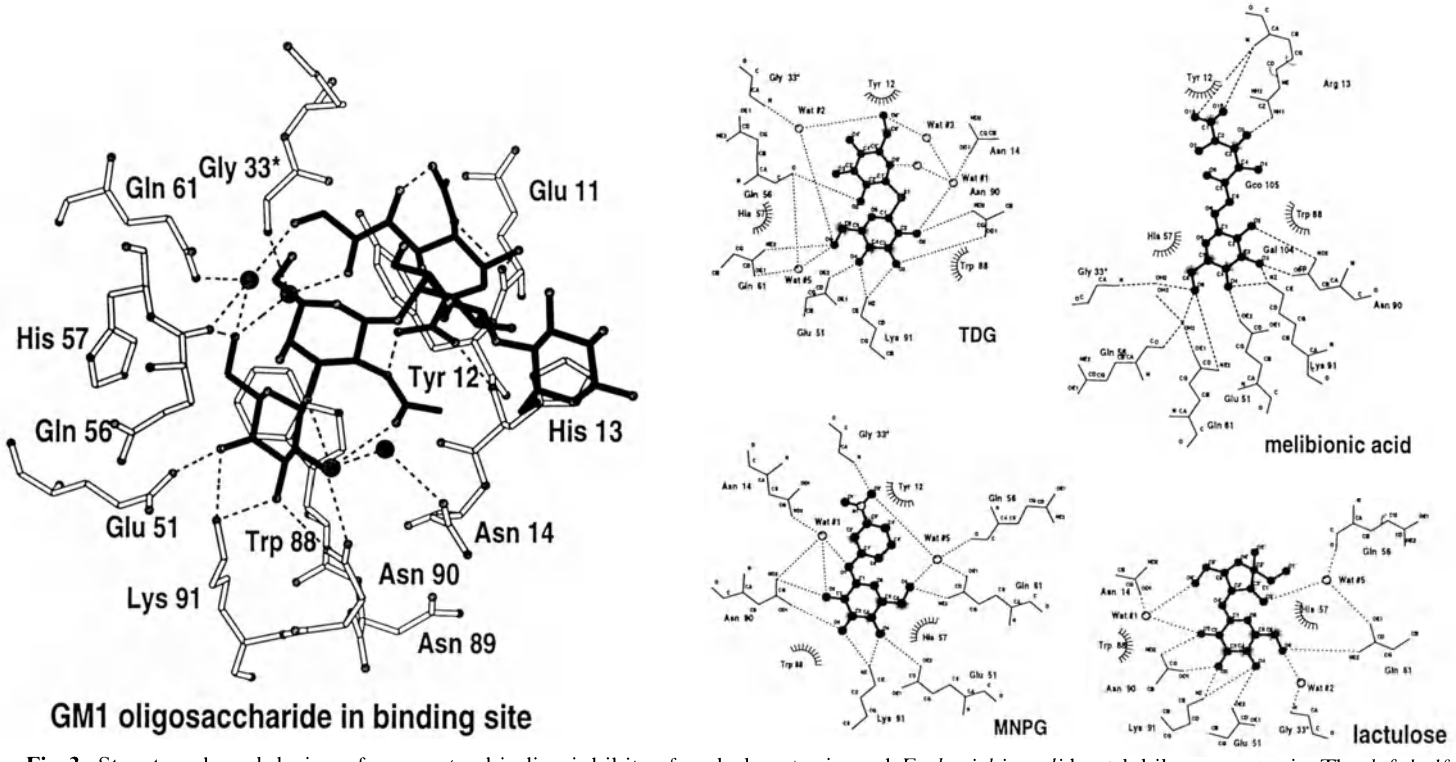

Fig. 3. Structure-based design of a receptor-binding inhibitor for cholera toxin and *Escherichia coli* heat-labile enterotoxin. The *left half* of the figure shows the pentasaccharide of the natural receptor, ganglioside GM1, bound to the cholera-toxin B pentamer (MERRITT et al. 1994; 1998). To the *right* are shown the binding interactions made by four small candidate inhibitors identified based on analysis of the toxin–receptor structure (MINKE et al., 1999). The structures of these candidate inhibitors, complexed to the *E. coli* toxin (MERRITT et al. 1997b), serve as the basis for further rounds of improved inhibitor design

F. Enzymatic Mechanism

Both CT and LT-I covalently modify $G_{s\alpha}$ (a trimeric G protein involved in signal transduction) by ADP–ribosylating the catalytically crucial $G_{s\alpha}$ residue Arg201, using nicotinamide adenine dinucleotide (NAD) as a substrate:

$$NAD^+ + G_{s\alpha} \rightarrow \text{ADP–ribose–Arg201-}G_{s\alpha} + \text{nicotinamide} + H^+ \quad (1)$$

The stereochemistry of this reaction has been determined for CT and LT-I and reveals that β-NAD is converted to α-ADP–ribose (Oppenheimer 1978; Moss et al. 1979), suggesting an S_N2 reaction mechanism. Two active-site residues have been identified as being probably important for catalysis. The first residue, Glu112, is definitely involved in catalysis and is strictly conserved in all ADP–ribosylating toxins (Cieplak et al. 1995). The second residue, His44, has been shown to be critical in CT, as the His44Asn mutation resulted in undetectable ADP–ribosylation of $G_{s\alpha}$ (Kaslow et al. 1992). Moreover, the equivalent histidine in PT, His35, has been shown to be important for the catalytic rate (Xu et al. 1994). It has been proposed that, in PT, LT, and CT, the histidine is involved in activating the acceptor moiety (which is a cysteine in PT and an arginine in CT and LT-I) by making a hydrogen bond to increase its nucleophilicity (Locht and Antoine 1995; Fig. 4a). The catalytic glutamic acid has been proposed to retrieve the hydrogen of the 2'-hydroxyl of NAD^+, thereby promoting the formation of an oxocarbonium-like intermediate (Locht and Antoine 1995). This intermediate would then weaken the *N*-glycosidic, making it susceptible to nucleophilic attack by the arginine. The His44-activated arginine would then cleave the *N*-glycosidic bond by an SN2-like mechanism, releasing nicotinamide and ADP–ribosyl–arginine.

I. Substrates, Artificial Substrates, and Inhibitors

The affinity of LT-I/CT for NAD is ~3 mM (Moss et al. 1976; Mekalanos et al. 1979a; Osborne et al. 1985; Larew et al. 1991), which is a factor of 100–1000 weaker than the NAD affinity for PT, exotoxin A (ETA), and diphtheria toxin (DT; Locht and Antoine 1995). Two hypotheses have been published that attempt to rationalize the weaker affinity of LT-I and CT. The first hypothesis is that the presence of two active-site tyrosines in DT and ETA, which are not present in LT or CT, may account for this difference (Domenighini et al. 1994). The second hypothesis is that the presence of loop residues 49–55, which occlude the active site in LT-I, might explain the much higher K_m for LT-I (Sixma et al. 1993). Quite interestingly, in the Arg7Lys mutant structure of LT (Van den Akker et al. 1995), this loop appeared to be completely flexible, demonstrating the ability of this loop to adopt conformations other than those observed in wild-type toxin. In addition to attacking arginine residues in $G_{s\alpha}$ and several other proteins, LT-I and CT can also ADP–ribosylate small artificial substrates. Several of those compounds, such as agmatine (Osborne et al. 1985), I-GT (Mekalanos et al. 1979), and diethylamino benzylidine–

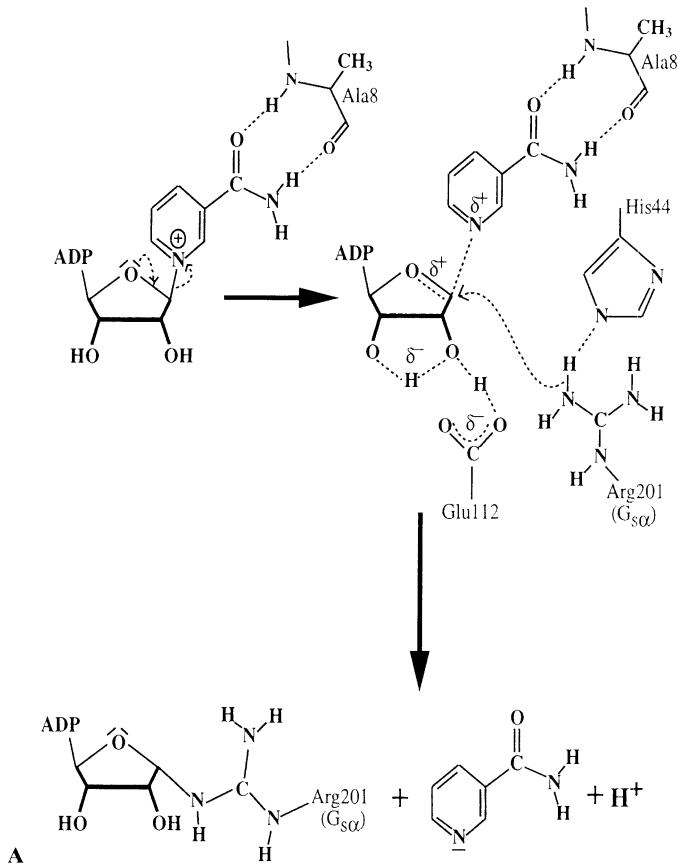

Fig. 4. A The proposed catalytic adenosine diphosphate–ribosylation mechanism of heat-labile enterotoxin (LT)-I. This mechanism is adopted from the proposed mechanism for pertussis toxin (LOCHT and ANTOINE 1995). Additional details about the interactions of the amide of nicotinamide with the backbone atoms of Ala8 are extrapolated from the nicotinamide adenine dinucleotide (NAD)-bound diphtheria toxin (DT) coordinates (BELL and EISENBERG 1996) and the exotoxin-A structure complexed to adenosine monophosphate and nicotinamide (LI et al. 1995). **B** Postulated NAD-binding mode in the active site of LT-I. The NAD-binding mode, shown in *thick black lines*, is derived from a superposition of the active sites of LT-I and DT. The superposition yielded a root-mean-squared deviation of 1.6 Å for 42 structurally equivalent Cα atoms. Residues that are mentioned in the text and residues that are postulated to interact with NAD (as extrapolated from the NAD-bound DT crystal structure) are shown.

aminoguanidine (NARAYANAN et al. 1989) are used in CT or LT-I enzymatic ADP–ribosylation assays. The substrate with the best affinity for CT characterized to date is *m*-iodobenzylguanidine, with a K_D of 6.5 μM (LOESBERG et al. 1990). At position Arg146, the A1 fragment can also serve as an acceptor for its own ADP–ribosyltransferase activity, but this auto-ADP–ribosylation has not been found to be physiologically relevant (LAI et al. 1983; OKAMOTO

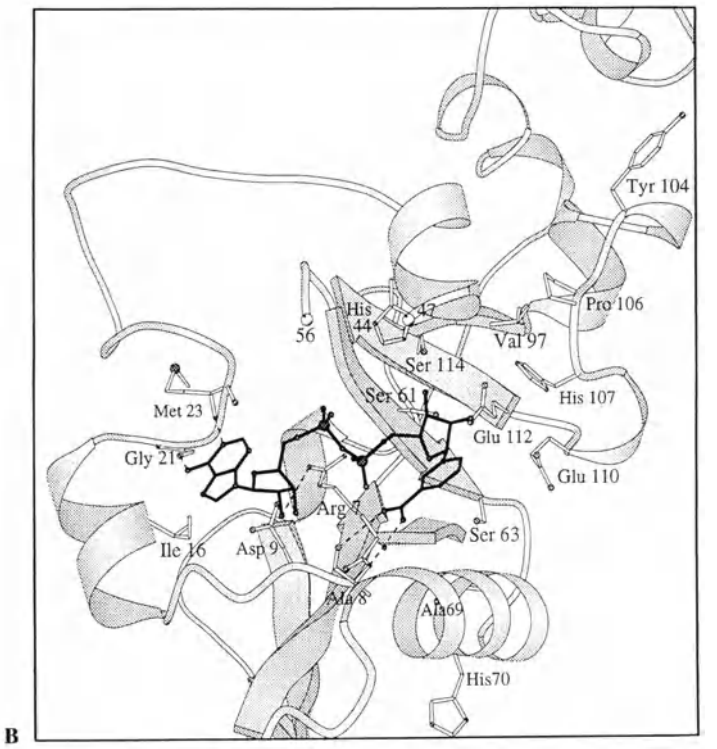

Fig. 4. *Continued*

et al. 1988). The peptide substrates poly-arginine (Lai et al. 1981) and kemptide, which has a K_m of 4.3 mM for CT (Kharadia et al. 1987), can also be ADP–ribosylated by CT.

Several NAD analogs are known to be substrates or inhibitors of LT-I and CT, although their affinities are quite low. For example, acetylpyridine–adenine dinucleotide has an inhibition constant of 17 mM (Galloway and Van Heyningen 1987), while the inhibitor carba-NAD has an inhibition constant of ~6 mM (Slama et al. 1989) and is, moreover, a challenge to synthesize.

II. NAD-Binding Site

The precise mode of binding of NAD to LT-I or CT remains unknown. However, two related toxins have been crystallized in the presence of NAD or NAD analogs. A DT structure has been determined in the presence of NAD (Bell and Eisenberg 1996). The structure of ETA has been determined, first in the presence of nicotinamide and AMP (Li et al. 1995) and, subsequently, complexed to the NAD analog β-methylene-thiazole-4-carboxamide adenine dinucleotide (Li et al. 1996). Binding of the NAD substrate or substrate analog causes some significant conformational changes in both toxins, but these are

mostly restricted to residues 458–463 in ETA and to residues 39–46 in DT (BELL and EISENBERG 1996, 1997). These regions become more disordered once NAD is bound. Whether an increase in local disorder upon NAD binding also occurs in LT-I or CT is not known, and biochemical data seem to indicate the opposite. It was observed that binding of NAD^+ causes the A1 subunit of CT to be unsusceptible to proteolysis by trypsin, suggesting that binding of NAD stabilizes the A1-subunit structure (GALLOWAY and VAN HEYNINGEN 1987). The disordered residues 39–46 in DT have been postulated to be involved in binding the second substrate, elongation factor 2 (BELL and EISENBERG 1996). The LT-I loop that corresponds to these residues in DT and ETA comprises residues 47–56, although the size, exact position, and sequence are different in the LT/CT family compared with the DT/ETA subfamily of toxins.

A superposition of the NAD-bound DT coordinates onto the LT-I structure yields a rough model for the mode of binding of NAD to LT-I and its postulated interactions in the active site (Fig. 4b). The loop 47–56 has been omitted from the figure, since it clashed sterically with the NAD model. Several hydrogen bonds between ligand and A subunit may be formed, based upon structurally equivalent atoms observed in the DT and ETA structures. The NAD model is also consistent with mutagenesis results in LT-I and CT, as discussed below.

Additional structural information about the active sites of LT-I and CT can be derived from extensive site-directed-mutagenesis data present in the literature (FEIL et al. 1998). Figure 4b shows active-site residues that have been mutated and that may be informative with respect to the postulated binding of NAD. Table 1 lists the biological effects of single-residue substitutions in the A-subunit of LT-1 or CT found to have a significant effect on either the toxicity or enzymatic activity.

The catalytic activity of the toxins is an obvious target for drug design; however, to date, the structural data to support such an effort is weaker than for blocking toxin assembly or receptor binding. The conformation of the catalytic site, as seen in the CT and LT-I holotoxin structures, is clearly not that of the active toxin during catalysis; in the observed conformation, there is insufficient room in the active-site cleft for substrate binding. The flexibility necessary for structural rearrangement to accommodate substrate binding may depend on the existence of internal cavities in the structure of the inactive conformation, such as that seen near residue Val97 (MERRITT et al. 1995). Based on the structure of the Arg7Lys mutant of LT-I, VAN DEN AKKER et al. (1995) have suggested that A-subunit residues 47–56 could be important for substrate recognition and that, in the fully active state, this loop must be displaced. Further support for this proposal comes from structural work on the related toxin LT-II, discussed below.

G. The LT-II Family

In the early 1980s, a new group of LT-IIs was discovered (GREEN et al. 1983; GUTH et al. 1986a). The *E. coli* strains that produced these new toxins were first

Table 1. Single-residue substitutions in the A-subunit of LT-1 or CT having a significant effect on either toxicity or enzymatic activity

Site	Mutation	References	Comments
Arg 7	Lys	Burnette et al. 1991; Lobet et al. 1991; Kaslow et al. 1992; Pizza et al. 1994	Residue Arg7 is involved in an extensive hydrogen-bond network with the carbonyl atoms of Val53, Arg54, Ser61, and the carboxyl group of Asp 9 at the bottom of the active site (Sixma et al. 1993). This Arg residue is homologous to His21 in DT and His440 in ETA, each of which are involved in a hydrogen-bond interaction with the ribose of the adenosine moiety of NAD (Li et al. 1995; Bell and Eisenberg 1996). Substitution of the Arg side chain will, thus, likely affect the ability of the toxin to bind NAD. The crystal structure of this mutant (Van den Akker et al. 1995) revealed a flexibility of loop 47–56
Asp 9	Glu/Lys	Kaslow et al. 1992; Vadheim et al. 1994	Asp9 is critical in that its side chain is likely also involved in an interaction with the ribose of the adenosine moiety of NAD in a manner similar to that of the structurally equivalent Thr23 and Thr442 in DT (Bell and Eisenberg 1996) and ETA (Li et al. 1996; Fig. 4), respectively
His 44	Asn	Kaslow et al. 1992	The histidine at position 44 is located near Glu112 in the LT-I structure (Fig. 4) and may be involved in orienting Glu112, modulating the catalytic properties of Glu112, or activating the acceptor substrate via a hydrogen-bond interaction, as suggested for the structurally equivalent His in PT (Locht and Antoine 1995), as discussed previously
Thr 50	Gly/Pro	Feil et al. 1996	Residue Thr50 is located in active-site loop 47–56. This loop is speculated to move out of the active site to accommodate the NAD substrate. The exact importance of Thr50 in the active-site loop is not known, but this residue could be involved in interacting with either of the two substrates (Feil et al. 1996)
Val 53	Asp/Glu/ Gly/Pro/ Tyr	Pizza et al. 1994; Fontana et al. 1995; Feil et al. 1996, in press	Val53 is located in loop 47–56 which, in order for NAD to bind in a manner similar to that in DT and ETA, must move out of the active site. Potential roles for Val53 are interaction with either NAD or the acceptor substrate
Ser 61	Phe/Thr	Harford et al. 1989; Cieplak et al. 1995a	Ser61 is located close to the postulated NAD-binding site, and the introduction of even an additional methyl group, as in a threonine, is not accommodated, resulting in loss of activity in the mutants. However, DT and ETA have evolved to incorporate a large tyrosine side chain at the structurally equivalent position

Table 1. *Continued*

Site	Mutation	References	Comments
Ser 63	Lys	PIZZA et al. 1994; FONTANA et al. 1995	This residue is located in the postulated binding pocket of the nicotinamide moiety of NAD (Fig. 4). Introduction of the larger and charged lysine side chain did not cause any structural rearrangement but probably hampers access by the NAD substrate (VAN DEN AKKER et al. 1997)
Val 97	Lys/Tyr	PIZZA et al. 1994; FONTANA et al. 1995	Val97 is located in a small hydrophobic cavity near the catalytic residue Glu112. Crystallographic analysis of the Val97Lys mutant revealed that the lysine moiety forms a salt bridge with Glu112 (MERRITT et al. 1995b). This salt bridge will, thus, most likely influence the properties of Glu112 in terms of its pKa. In addition, it might also restrict the Glu112 side chain from adopting the required conformation(s) needed for catalysis or limit access of the acceptor substrate
Tyr 104	Asp/Lys/ Ser	PIZZA et al. 1994; FONTANA et al. 1995	Tyr104 is located in a loop distant from the active site and NAD-binding site (Fig. 4). This residue has been postulated to be involved in binding Gsα (Merritt et al. 1996)
Pro 106	Ser	FONTANA et al. 1995	Pro106 is located in a loop quite distant from the active site (Fig. 4). The Cα of residue 106 is 10.6 Å from the Cα of Glu112. This residue has been postulated to be involved in binding Gsα (MERRITT et al. 1996)
His 107	Glu	PIZZA et al. 1994	His107 is located near Glu112 (Fig. 4). The imidizole ring of residue 107 is 5.5 Å from the carboxyl moiety of Glu112. Changing His107 to Glu may affect the characteristics of the catalytic Glu112 or its nearby environment
Glu 110	Asp/Ser	LOBET et al. 1991; PIZZA et al. 1994; CIEPLAK et al. 1995a	Glu110 is located near the catalytic Glu112 (Fig. 4). The exact role of Glu110 is not known, but the residue may be involved in catalysis or Gsα binding. The fact that Glu110 cannot be replaced, even by an Asp, implies that the position of the carboxyl group is extremely critical for the activity of the toxin
Glu 112	Ala/Asp/ Gln/Lys	TSUJI et al. 1990, 1991; LOBET et al. 1991; KASLOW et al. 1992;	Glu112 is one of the two completely conserved residues identified (Gly21 was also found to be conserved in the family of the ADP–ribosylating toxins; BELL and EISENBERG 1996). The equivalent Glu in DT, ETA, and PT has been shown (by photolabeling experiments

		Pizza et al. 1994; Cieplak et al. 1995a	using radiolabeled NAD) to be near the NAD-binding site (Carroll and Collier 1984, 1987; Barbieri et al. 1989). The role of the conserved Glu in catalysis has also been established for these related toxins (Douglas and Collier 1990; Wilson et al. 1990; Antoine et al. 1993). Recently, evidence has also been found for a role of Glu112 in catalysis in LT-I (Cieplak et al. 1995a)
Ser 114	Glu/Lys	Pizza et al. 1994; Fontana et al. 1995	Ser114 is located in close proximity to the postulated NAD-binding pocket and Glu112. The introduction of a larger and charged side chain at position 114 may affect NAD binding or the catalytic properties of Glu112
Residues of inconclusive importance for the biological activity of LT-I and CT			
Ala 69	Gly/Val	Cieplak et al. 1995a	Substitution of Ala69 caused only a moderate decrease in ADP–ribosyltransferase activity, using agmatine as a substrate (Cieplak et al. 1995a). Ala69 is located on the large helix in the active site and is mostly buried (Fig. 4b). It is likely that the relatively small difference in side-chain character and volume between Gly or Val and the wild-type Ala can be accommodated by minor conformational changes that have little effect on activity
His 70	Asn/Pro	Kaslow et al. 1992; Cieplak et al. 1995a	His70 has been postulated to bind Gsα (Burnette et al. 1995), because mutating the residue to Asn caused the ADP–ribosylation of Gsα to drop to 10%, while the ADP–ribosylation of other proteins was unaffected (Kaslow et al. 1992). However, a more recent mutagenesis study claims to rule out any important role for His 70 (Cieplak et al. 1995a)
Arg 192	Gly	Grant et al. 1994; Dickinson and Clements 1995	Arg192 is near or at the known cleavage site for activation of LT-I and CT, which can readily be cleaved by trypsin (Spicer and Noble 1982). Since the specificity of trypsin is to cleave after an Arg, the Arg192Gly mutation was designed to make the A subunit unsusceptible to proteolysis, thereby potentially preventing it from becoming activated. Initially, it was reported that the Arg192Gly substitution caused only a delay in the ability to increase intracellular levels of cAMP in Caco-2 cells and that it had no drastic effect on the biological and enzymatic activities of the toxin (Grant et al. 1994). However, another group reached different conclusions and found negligible activity in mouse Y-1 adrenal tumor cells and no detectable ADP–ribosyltransferase activity upon introduction of the Arg192Gly mutation (Dickinson and Clements 1995)

ADP, adenosine diphosphate; *cAMP*, cyclic adenosine monophosphate; *CT*, cholera toxin; *DT*, diphtheria toxin; *ETA*, exotoxin A; *LT*, heat-labile enterotoxin; *NAD*, nicotinamide adenine dinucleotide; *PT*, pertussis toxin.

isolated from feces of a water buffalo in Thailand (PICKETT et al. 1986) and were later isolated from feces of patients with diarrhea in Sao Paulo and from food (GUTH et al. 1986a). Recent studies suggest that LT-II-producing *E. coli* are more common in cattle and buffalo than in humans (SERIWATANA et al. 1988; CELEMIN et al. 1994). LT-IIs have not (yet) been connected with any diarrheal disease in either animals or humans and also do not cause secretion in ligated rabbit ileal segments at doses comparable to those of CT controls (HOLMES et al. 1986). Despite this, in many assays, LT-IIs behave very much like the toxins LT-I and CT. LT-IIs bind to Y1 mouse adrenal cells and intestinal cells (DONTA et al. 1992). LT-IIs have been shown to be toxic to mouse Y1 adrenal cells (GUTH et al. 1986b), causing rounding of Y1 adrenal cells, accompanied by increased levels of intracellular cAMP (HOLMES et al. 1986). In addition, LT-IIs have been shown to ADP–ribosylate $G_{s\alpha}$ (LEE et al. 1991) and activate (by ADP–ribosylation) adenylate cyclase in human fibroblasts (CHANG et al. 1987), suggesting a mode of action similar to those of CT and LT-I.

Like LT-I, LT-IIs contain an A subunit [molecular weight (M_r) = 28000 Da] and five B subunits (M_r = 11800 Da). The A subunit can be cleaved by trypsin yielding an A1 (M_r = 21000 Da) and A2 (M_r = 7000 Da) fragment (GUTH et al. 1986b). The overall sequence identity of the A subunits of LT-IIa and LT-IIb is 71% and, for the B subunits, the identity is 66% (PICKET et al. 1987, 1989). The LT-IIa and LT-IIb A-subunit sequences are quite similar to that of LT-I (VAN DEN AKKER et al. 1996b), as the sequence identity between the LT-IIs and LT-I is about 55% (PICKET et al. 1989). In contrast, no homology could be detected when comparing the LT-IIa and LT-IIb amino acid sequence with the LT-I and CT B-subunit sequences (PICKET et al. 1989). Given the limited sequence similarity between LT-I and LT-IIs, it is not surprising that they do not cross-react in neutralization or immunodiffusion tests with anti-LT/CT antibodies (GUTH et al. 1986b). Considerable variation in heterogeneity among B-subunit genes from different LT-II producing strains has been suggested from hybridization studies (PICKET et al. 1987, 1989). Despite the very different B-subunit amino acid sequences, LT-IIa and LT-IIb A subunits and LT-I B subunits can form hybrid toxins (although with low efficiency; CONNELL and HOLMES 1992b).

Crystallographic study shows the LT-IIb holotoxin structure to be very similar to those of LT-I and CT for both the A and B subunits (VAN DEN AKKER et al. 1996b). The degree of structural homology in the B subunits is unexpectedly high, given the lack of sequence similarity previously identified. Retrospective analysis of the LT-IIb sequence after structural alignment with LT-I and CT identified 11 conserved residues in the 99-residue B subunit (VAN DEN AKKER et al. 1996b). The catalytic domain of the A subunit of LT-IIb is structurally quite similar to that of LT-I, although its orientation with regard to the B pentamer differs by a rotation of 24°. This difference is partly due to a longer A2 helix in LT-IIb, which extends more deeply into the pore of the B pentamer (and at a steeper angle) than does the corresponding A2 helix of LT-I. In this regard, the LT-IIb holotoxin is more similar to CT than to LT-I (Fig.

1). Within the A-subunit catalytic domain, the region of greatest difference between LT-IIb and LT-I is found for the loop consisting of residues 24–34.

The active-site loop consisting of residues 47–56, implicated in substrate binding (Van den Akker et al. 1995; Feil et al. 1998), is found in LT-IIb in the same inactive conformation (blocking the active site) as the homologous loop in wild-type LT-I structures. Sequence differences between LT-I and LT-IIb in this loop and elsewhere are sufficient to confer differing substrate specificities; small guanidium-containing compounds like agmatine that are good substrates for LT-I are very poor substrates for LT-IIa and LT-IIb (Lee et al. 1991). Mutational studies of the residues in the 47–56 loop (Feil et al 1998) support its importance in substrate recognition and catalytic activity.

The lack of sequence similarity between the B subunits of LT-I and LT-IIs is reflected in the differences in ganglioside specificity among these toxins. Unlike LT-I, LT-IIa is not very specific and recognizes a wide variety of gangliosides, including GM2, GM1, GD1a, GD2, GD1b, GT1b, and GQ1b (Fukuta et al. 1988). LT-IIb binds to GD1a, GT1b, and (weakly) to GM3 and GM2 (Fukuta et al. 1988). The hydroxyl groups of Ser13 and Ser14 of the B subunits in both LT-IIa and LT-IIb have been shown to be critical for the toxins' ganglioside affinity (Connell and Holmes 1992a, 1995). The N-terminal half of the B subunits of LT-IIa and LT-IIb have been found to contain the toxins' ganglioside-binding activities (Connell and Holmes 1995). Clearly, more studies are needed to unravel the functional properties of the LT-II toxins at the structural level.

H. Perspectives

Crystallographic studies of cholera and related enterotoxins have revealed a wealth of information on the molecular interactions involved in toxin architecture, receptor binding, and catalytic activity. In conjunction with biochemical investigations of toxin properties, such as substrate specificity and mutational analysis, crystallographic studies can provide a solid basis for the design of biologically active small molecules targeting specific aspects of normal-toxin mechanism. Work is already underway to design inhibitors of toxin assembly, receptor binding, and catalysis. In the future, detailed structural knowledge may also contribute to increased understanding of other properties of these toxins, such as their remarkable immune-system-stimulating abilities. The latter aspects will be discussed in Chap. 7.

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CHAPTER 7

Mechanism of Cholera Toxin Action: ADP-Ribosylation Factors as Stimulators of Cholera Toxin-Catalyzed ADP-Ribosylation and Effectors in Intracellular Vesicular Trafficking Events

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A. Introduction

More than 100 years after the recognition that *Vibrio cholerae* causes the deadly diarrhea characteristic of cholera (KOCH 1884), the devastating effects of this disease continue to be felt throughout the world (MATHAN 1998). Efforts to prevent the disease rely heavily on measures intended to improve basic hygiene and to ensure sanitary water and food supplies (KUMATE 1997). Rehydration (to combat the lethal dehydration) continues to be the cornerstone of treatment protocols (DUGGAN 1998). Alternative therapies include the use of antimicrobials, e.g., ciprofloxacin (KHAN et al. 1996), and antisecretagogues, e.g., the Ca^{2+}-channel blocker nifedipine (TIMAR-PEREGRIN et al. 1997), the enkephalinase inhibitor acetorphan (HINTERLEITNER et al. 1997), the somatostatin analogue octreotide (BARDHAN et al. 1994) and the K^{+}-channel blocker clotrimazole (RUFO et al. 1997). Vaccines derived from attenuated *V. cholerae,* from recombinant cholera toxin (CT) constructs, or from CT fragments have had varying efficacies in field trials (MEKALANOS and SADOFF 1994; FINKELSTEIN 1995; HOLMGREN and SVENNERHOLM 1996; WALDOR and MEKALANOS 1996b; LEVINE 1997; ROBERTSON et al. 1997). The emergence of new *V. cholerae* strains (MEKALANOS et al. 1997), possibly due to the lateral transfer of CT genes via the filamentous bacteriophage CTX (WALDOR and MEKALANOS 1996a) to nontoxinogenic *V. cholerae* strains, and the multiplicity of factors recognized as being involved in cholera pathogenesis (MEKALANOS et al. 1997) complicate antigen choice and, ultimately, the effectiveness of the vaccine.

CT, a major virulence factor of *V. cholerae*, is but one of several proteinaceous toxins known to be secreted by pathogenic *V. cholerae* (KAPER et al. 1995). The original model of intestinal epithelial cell intoxication by CT (resulting in adenylyl cyclase activation, which leads, in turn, to increased cyclic adenosine monophosphate (cAMP) concentration and the opening of Cl^{-} channels; FINKELSTEIN 1988) has become more complex. Intoxication includes the actions of other signaling molecules, such as prostaglandin E_2, platelet-activating factor (GUERRANT 1994), and 5-hydroxytryptamine (KAPER et al. 1995). Moreover, local secretory responses in the intestine are compounded

by distant responses propagated by the enteric nervous system (Nocerino et al. 1995; Lundgren and Jodal 1997). In addition to yielding clues to the pathogenesis and treatment of cholera, the study of CT has already led to an understanding of complex signaling events involving heterotrimeric guanosine triphosphate (GTP)-binding proteins (Moss and Vaughan 1993), the identification of several new families of molecules [i.e., adenosine diphosphate (ADP)–ribosylation factors and the regulators thereof (Moss and Vaughan 1998), and eukaryotic mono-ADP ribosyltransferases (Okazaki and Moss 1998)], and the definition of CT adjuvant effects on mucosal immune responses (Snider 1995; Sabbaj et al. 1997; Hirst et al. 1998; Oliver and Elson 1998).

CT and the very similar heat-labile toxin from toxigenic *E. coli* (LT) not only contain the machinery needed to exploit endogenous molecules for their cell-surface attachment but, once bound, the subsequently internalized toxins enzymatically modify several proteins with ADP ribose, including the positive regulator of adenylyl cyclase, $G_{\alpha s}$. The ADP ribosylation factors (ARFs) are 20-kDa guanine-nucleotide-binding proteins that are the major *in vitro* stimulators of CT/LT-catalyzed ADP-ribosylation (Fig. 1) and have been shown to be involved in an array of cellular processes, all of which appear to be related to the control of vesicle formation and trafficking (Donaldson et al. 1995; Patton et al. 1997a). However, it remains to be shown whether ARF encounters CT/LT inside the cell and plays a role in CT/LT action.

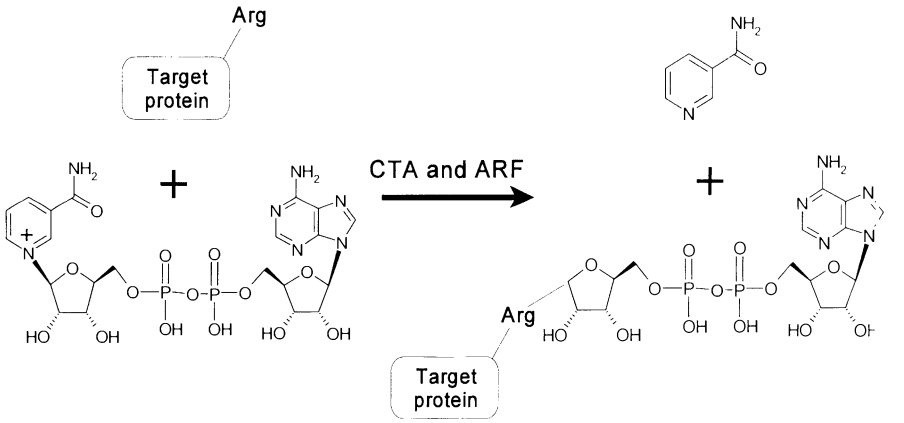

Fig. 1. Cholera toxin-catalyzed adenosine diphosphate (ADP)-ribosylation. ADP ribosylation factors stimulate the ADP-ribosyltransferase activity of cholera toxin *in vitro*. Cholera toxin action on arginine-containing target proteins (the positive regulator of adenylyl cyclase, G_s) results in formation of the α-anomeric, ADP-ribosylated product and free nicotinamide from β-nicotinamide adenine dinucleotide cation. The exact chemical linkage formed in the ADP-ribosylated product is described elsewhere (Oppenheimer 1978). Free arginine and simple guanidino compounds (agmatine) also serve as substrates in this reaction.

In addition to discussing the enzymatic nature of CT and LT, this review will focus on the structural and functional characteristics of the ARF family of proteins and on effectors of ARF function, molecules that ARFs regulate, and current thoughts on ARF cellular functions.

B. Cholera Toxin

I. Structure

CT is an 85-kDa protein belonging to the AB_5 toxin family (Merritt and Hol 1995). There is little similarity in primary structure among AB_5-family members, with the exception of LT and CT, which are 80% identical in amino acid sequences and are postulated to have evolved from a common ancestor, because of conservation of activity (Yamamoto et al. 1984) and function (Lee et al. 1991). All members of the AB_5 family are characterized by a structure in which a catalytic A subunit (CTA) is non-covalently associated with a pentamer of B subunits. CTA consists of a catalytic A1 chain (22 kDa) linked by a single disulfide bond to an A2 chain (5 kDa); CTB is a homopentamer of 11.6-kDa B subunits and binds to the cell-surface receptor, ganglioside GM1. Crystal structures revealed that CTA, like LT (Sixma et al. 1993), is anchored above the ring-like structure of the CTB pentamer by insertion of the A2 chain into the "central pore" in CTB (or choleragenoid) formed by the five aligned B chains (Zhang et al. 1995b). The intact AB_5 structure forms what is known as CT or LT holotoxin. The existence of fine differences between LT and CT are noted throughout the structures, but the most pronounced difference is in the A2 subunit. In both holotoxins, the A_2 chains form an elongated helix that is in close contact with the A1 subunit, but the portion of LTA2 that penetrates its B pentamer is an elongated chain (Sixma et al. 1993), whereas the corresponding portion of CT is a helix throughout its length (Zhang et al. 1995b). As a detailed discussion of existing toxin structural data is beyond the scope of the review, the reader is referred to additional papers in this volume and elsewhere (Merritt et al. 1994a, 1994b, 1994c, 1995, 1997, 1998; Verlinde et al. 1994; Van den Akker et al. 1997).

II. Biochemistry

The early recognition that nicotinamide adenine dinucleotide (NAD) is required for CT-catalyzed activation of adenylyl cyclase (Gill 1975) led to the identification of CT as an arginine-specific ADP ribosyltransferase (Moss and Vaughan 1977b). To generate active CT or CTA, the A chain must be proteolytically cleaved between residues 192 and 195 (Moss et al. 1976; Mekalanos et al. 1979b), thus forming the A1 and A2 chains (native CT, purified from *V. cholerae*, is usually "nicked" by endogenous proteases). The disulfide bond (between Cys 187 and Cys 199) linking the A1 and A2 chains must also be reduced (Mekalanos et al. 1979b; Tomasi et al. 1979). The A1 chain catalyzes

transfer of the ADP-ribose portion of NAD to the guanidino portion of an arginine acceptor (which, depending on the reaction conditions, can be part of another protein; GILL and MEREN 1978; JOHNSON et al. 1978; KASLOW et al. 1980; NORTHUP et al. 1980; ABOOD et al. 1982; VAN DOP et al. 1984), to an arginine within the toxin itself (i.e., auto-ADP ribosylation; TREPEL et al. 1977), or to free arginine (or other simple guanidino compounds; MOSS and VAUGHAN 1977b; MEKALANOS et al. 1979a; TAIT and NASSAU 1984). In the transferase reactions, CTA1 utilizes β-NAD to generate an α-anomeric product (OPPENHEIMER 1978). CTA1 can also transfer ADP-ribose to water (Moss et al. 1976, 1978, 1979) and, thus, is also an NAD glycohydrolase or NADase. Assays to measure each of the above activities of CT and LT have been described elsewhere in detail (PATTON et al. 1997b).

The presence of a critical glutamate residue at or near the NAD^+-binding and catalytic sites of CT and LT, is one similarity that CT and LT share with the ADP-ribosylating toxins from *Corynebacterium diphtheriae* (diphtheria toxin or DT; CARROLL and COLLIER 1984), *Bordetella pertussis* (pertussis toxin or PT; BARBIERI et al. 1989) and *Pseudomonas* (exotoxin A or ETA; CARROLL and COLLIER 1987). In LT, replacement of Glu112 with Lys (TSUJI et al. 1990, 1991) or Asp (LOBET et al. 1991) resulted in toxins that lacked or had greatly reduced ADP-ribosyltransferase activity. Consistent with the function of Glu112 as an active-site residue, an LT Glu112Lys mutant retained its ability to interact with ADP-ribosylation factor (discussed below; Moss et al. 1993). The position of Glu112 in CTA1 and LTA1, as determined from models generated using both crystallographic (SIXMA et al. 1993; ZHANG et al. 1995b) and computer-modeling methods (DOMENIGHINI et al. 1994), accords with its role as a critical active-site residue.

The major CT and LT substrate in cells is $G_{\alpha s}$, the α subunit of the heterotrimeric GTP-binding protein complex known to stimulate adenylyl cyclase (GILL and MEREN 1978; JOHNSON et al. 1978; KASLOW et al. 1980; NORTHUP et al. 1980). In resting cells, $G_{\alpha s}$ exists in a guanosine diphosphate (GDP)-bound state complexed with $G_{\beta\gamma}$ on the plasma membrane. When stimulated by an external signal via a membrane-bound receptor, $G_{\alpha s}$ becomes GTP bound and dissociates from $G_{\beta\gamma}$; $G_{\alpha s}$-GTP then stimulates adenylyl cyclase. ADP-ribosylation of $G_{\alpha s}$ not only causes the release of $G_{\alpha s}$ from $G_{\beta\gamma}$ (KAHN and GILMAN 1984a) but also accelerates the release of GDP from $G_{\alpha s}$ (BURNS et al. 1982) and inhibits the ability of $G_{\alpha s}$ to hydrolyze GTP and return to its inactive state (CASSEL and SELINGER 1977; JOHNSON and BOURNE 1977; NAVON and FUNG 1984). Thus, persistently activated $G_{\alpha s}$ can lead to high levels of cAMP production that are known to be associated with CT action in cells.

III. Toxin Internalization

It is now apparent that portions of the CT-internalization pathway may differ between polarized and nonpolarized cell types (SANDVIG et al. 1997) and that CT and LT may differ in their uptake and processing mechanisms. Work with

CT in intestinal epithelial cells is currently defining what are perhaps the critical events that occur during the pathogenesis of disease by *V. cholerae*. Intact holotoxin is required for intoxication of cells. Although CTB can enter cells, enzymatically active CTA cannot exert its effects on cells independently of CTB. CT binds to the apical surface of intestinal epithelial cells via ganglioside GM1-binding sites in each of the five B chains (FISHMAN 1982; MERRITT et al. 1994b), with the A-subunit oriented away from the plasma membrane (ORLANDI and FISHMAN 1993). There is a characteristic delay between the time of toxin binding and the activation of adenylyl cyclase, during which several events are thought to occur.

Toxin binding is followed by rapid endocytosis and proteolytic processing of LT and non-nicked CT (LENCER et al. 1997). Vesicular transport of the toxin is thought to occur (LENCER et al. 1992), leading to transcytosis of toxin to the basolateral membrane (LENCER et al. 1995b) for activation of adenylyl cyclase. Recently, CT was shown to be internalized through caveolae-like membrane domains (ORLANDI and FISHMAN 1998; WOLF et al. 1998); it is thought that the ability of ganglioside GM1 to associate with these structures ultimately controls toxin-mediated signal transduction (WOLF et al. 1998).

The fungal metabolite brefeldin A (BFA) inhibits an early transport step that is critical for the reduction (and thus activation) of A1 (LENCER et al. 1993). The roles of the retrograde transport pathway from the Golgi to the endoplasmic reticulum (ER) and for KDEL and RDEL sequences of CT and LT, respectively, in intestinal epithelial cells (CIEPLAK et al. 1995; LENCER et al. 1995a) or other epithelial cells (BASTIAENS et al. 1996; MAJOUL et al. 1996, 1998; SANDVIG et al. 1996) remain to be defined but may be important for maximal biologic activity (LENCER et al. 1995a). It is not clear at what point in the pathway the catalytic A1 subunit is exposed to cytosol, but this appears to occur late in the lag phase via a temperature-sensitive step (LENCER et al. 1992). Subsequent to the activation of adenylyl cyclase, there are additional signaling events that occur in the intestinal epithelial cells (and in the intestine generally) that are less well understood. There are most certainly differences between the mechanisms of CT- and LT-mediated events, based on the differences in clinical severity of the corresponding diseases. Of interest in that regard is the recent report of TURVILL et al. (1998) describing 5-hydroxytryptamine involvement in CT- (but not LT)-induced secretion.

C. ADP-Ribosylation Factors

I. Discovery of ARFs

Investigation of the nature of CT-catalyzed ADP-ribosylation of $G_{\alpha s}$ revealed that tissue factors (ENOMOTO and GILL 1979, 1980; PINKETT and ANDERSON 1982; SCHLEIFER et al. 1982) and GTP (MOSS and VAUGHAN 1977a; LIN et al. 1978; ENOMOTO and GILL 1979; NAKAYA et al. 1980; WATKINS et al. 1980) were

required for this activity. One of the tissue factors purified from rabbit-liver membranes was identified [by sodium dodecyl sulfate (SDS) polyacrylamide-gel electrophoresis] as a 21.5-kDa protein doublet (Kahn and Gilman 1984b) that supported CT-catalyzed ADP ribosylation of $G_{\alpha s}$ in the presence of GTP but not GDP (Kahn and Gilman 1986) and was thus named ADP-ribosylation factor or ARF. GTP, GDP, and their corresponding non-hydrolyzable analogues bound to ARF, but ARF hydrolysis of bound GTP was not detected (Kahn and Gilman 1986). Guanine-nucleotide binding was enhanced by 3 mM dimyristoyl phosphatidyl choline (DMPC) and 2 mM Mg^{2+}, whereas adenine nucleotides did not bind under any conditions (Kahn and Gilman 1986) Subsequently, a membrane-bound ARF (Tsai et al. 1987) and two soluble ARFs (Tsai et al. 1988) with biochemical properties similar to those described earlier were identified in bovine brain.

II. Biochemical Characterization of ARFs

ARFs act as allosteric activators of CTA1 *in vitro* (Tsai et al. 1987, 1988: Noda et al. 1990). As shown with native bovine ARF3, ARFs lower the K_m for both NAD and agmatine (Noda et al. 1990). Lipids and detergents not only enhance the ability of ARF to bind GTP (Bobak et al. 1990) or the non-hydrolyzable analogue GTPγS (Murayama et al. 1993) but, as was shown with 0.003% SDS (Noda et al. 1990), they cause an additional decrease in K_m and increase the V_{max} of the CT-catalyzed reaction. The effects of lipid and detergent on ARF activation of CTA might be explained by the observation that stable complexes of native bovine ARF3 or recombinant bovine ARF1 and CTA1 have been isolated only in the presence of 0.003% SDS (Tsai et al. 1991) or azolectin/cholate (Franco et al. 1993), respectively, and only when using gel filtration. Thus, lipids, detergents, and ARF appear to play a role in enhancing CTA activity in a cellular or physiological environment. The optimal temperature for CTA-catalyzed ADP-ribosylation of agmatine without lipid or ARF was 25–30°C and was shifted toward 37°C with addition of native bovine ARF3 and 3 mM DMPC/0.2% cholate (Murayama et al. 1993).

GTP binding to ARF is required for all known ARF activities. Normally, ARFs are isolated from native or recombinant sources in the GDP-bound, inactive state; in this state, ARFs are generally soluble and are generally isolated from cell supernatants. The release of GDP and binding of GTP causes ARF activation and binding to membranes (Kahn et al. 1991; Regazzi et al. 1991; Walker et al. 1992). Some data suggest that ARF is "loosely" associated with lipid membranes in the GDP-bound state (Franco et al. 1995) and that activation (GTP binding) promotes "tighter" binding. A "loose" association would allow ARF to sense the lipid environment before subsequent binding and may even influence membrane binding by the presence or absence of a particular lipid or perhaps by influencing nucleotide exchange on the ARF. In support of this concept, the binding of soluble native ARF from PC-12 cells to phosphatidylinositol or cardiolipin vesicles in a centrifugation assay was not

dependent on GTPγS addition to the assay, whereas the binding to phosphatidylserine vesicles was GTPγS-dependent (WALKER et al. 1992).

Transition from soluble to membrane-bound or from "loosely" membrane-associated to "tightly" membrane-associated forms upon GTP binding is thought to be mediated by a conformational change that affects the position of the ARF N-terminus (RANDAZZO et al. 1995). The ARF amphipathic N-terminal α-helix (AMOR et al. 1994) is modified by the co-translational addition of a myristic acid group to the N-terminal glycine (KAHN et al. 1988; KUNZ et al. 1993). It is thought that, in the GDP-bound form, the helix and the myristate are in close proximity to the ARF. However, when GTP is bound, the helix and fatty acyl moiety are oriented away from the protein in a manner that would facilitate their interaction with a membrane or other molecule, e.g., an ARF guanine nucleotide-exchange protein or GEP (FRANCO et al. 1995, 1996). This is partially because the exposure of the fatty acid group to solution would not be enthalpically favorable.

III. ARF Structure

1. The Primary Structures of ARFs

As ~180-amino acid proteins that are ubiquitous in eukaryotes, ARF proteins exhibit a high percentage of sequence identity, an indication that their functions have been well-conserved throughout evolution. ARF family members are continuously being added to sequence databases, but they can only truly be designated as ARFs if they have the ability to rescue the lethal yeast *arf1*$^-$, *arf2*$^-$ double mutant (KAHN et al. 1991), are capable of stimulating the ADP-ribosyltransferase activity of CTA1 (with $G_{\alpha s}$ or agmatine as an acceptor) and can stimulate ARF–dependent phospholipase D (PLD). It is not established, however, that all ARFs, e.g., those from non-mammalian sources, activate PLD. If a protein of plausible structure does not meet these requirements, it is designated an ARF-like protein (ARL). Thus, many newly recognized ARF family members should be grouped with ARLs (see below) until their activities are defined.

As shown in Fig. 2, the six known mammalian ARFs fall into three distinct classes based on amino acid and DNA sequence similarities and gene structure. It is becoming possible to relate these classes to cellular localization and function. Utilizing sequence alignment or dendritic-tree analysis, ARFs from other species can also be grouped into the three classes. In addition to a glycine at position 2, which is the site of the covalent attachment of myristic acid, the most highly conserved ARF sequences are those known to be critical for GTP binding and hydrolysis. These regions are well conserved throughout the families of 20-kDa GTP-binding proteins and the α subunits ($G_{\alpha s}$) of heterotrimeric GTP-binding proteins (MOORMAN et al. 1999). Region I, with the consensus sequence GX_4GK, is critical for phosphate binding and nucleotide hydrolysis. Region II (DX_2G) participates in Mg^{2+} and nucleotide-

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    ARF1  MGNIFANLFKGLFGKKEMRILMVGLDAAGKTTILYKLKLGEIVTTIPTIGFNVETVEYKN 60
    ARF3  MGNIFGNLLKSLIGKKEMRILMVGLDAAGKTTILYKLKLGEIVTTIPTIGFNVETVEYKN 60
 Class I  *****.**:*.*:**************************************************
    ARF4  MGLTISSLFSRLFGKKQMRILMVGLDAAGKTTILYKLKLGEIVTTIPTIGFNVETVEYKN 60
    ARF5  MGLTVSALFSRIFGKKQMRILMVGLDAAGKTTILYKLKLGEIVTTIPTIGFNVETVEYKN 60
Class II  ****:*:****:**************************************************
    ARF6  MG----KVLSKIFGNKEMRILMLGLDAAGKTTILYKLKLGQSVTTIPTVGFNVETVTYKN 56

    ARF1  ISFTVWDVGGQDKIRPLWRHYFQNTQGLIFVVDSNDRERVNEAREELMRMLAEDELRDAV 120
    ARF3  ISFTVWDVGGQDKIRPLWRHYFQNTQGLIFVVDSNDRERVNEAREELMRMLAEDELRDAV 120
 Class I  ************************************************************
    ARF4  ICFTVWDVGGQDRIRPLWKHYFQNTQGLIFVVDSNDRERIQEVADELQKMLLVDELRDAV 120
    ARF5  ICFTVWDVGGQDKIRPLWRHYFQNTQGLIFVVDSNDRERVQESADELQKMLQEDELRDAV 120
Class II  ************:*****:********************:** ********  *******
    ARF6  VKFNVWDVGGQDKIRPLWRHYYTGTQGLIFVVDCADRDRIDEARQELHRIINDREMRDAI 116

    ARF1  LLVFANKQDLPNAMNAAEITDKLGLHSLRHRNWYIQATCATSGDGLYEGLDWLSNQLRNQ 180
    ARF3  LLVFANKQDLPNAMNAAEITDKLGLHSLRHRNWYIQATCATSGDGLYEGLDWLANQLKNK 180
 Class I  ************************************************:***:*:
    ARF4  LLLFANKQDLPNAMAISEMTDKLGLQSLRNRTWYVQATCATQGTGLYEGLDWLSNELSER 180
    ARF5  LLVFANKQDMPNAMPVSELTDKLGLQHLRSRTWYVQATCATQGTGLYDGLDWLSHELSER 180
Class II  **:******:****.:**:******* **.*****************:******:****.*
    ARF6  ILIFANKQDLPDAMKPHEIQEKLGLTRIRDRNWYVQPSCATSGDGLYEGLTWLTSNYKS- 175

    ARF1  K 181
    ARF3  K 181
 Class I  *
    ARF4  -
    ARF5  -
Class II  -
    ARF6  -
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Fig. 2. Alignment of human adenosine diphosphate–ribosylation-factor (hARF)-deduced amino acid sequences. Sequences were aligned using ClustalX for the personal computer (ftp://ftp.ebi.ac.uk/pub/software/dos/clustalw/clustalx). References and accession numbers for GenBank (GB) and SwissProt (SP) databases are: hARF1 (Bobak et al. 1989, Peng et al. 1989) GB-416637 SP-P32889; hARF3 (Bobak et al. 1989) GB-114125 SP-P16587; hARF4 (Monaco et al. 1990) GB-114123 SP-P18085; hARF5 (Tsuchiya et al. 1991) GB-114127 SP-PP26437; hARF6 (Tsuchiya et al. 1991) GB-114129 SP-P26438. hARFs 1 and 3 constitute class-I ARFs, hARFs 4 and 5 are class-II ARFs, and hARF6 is the only class-III ARF. ARFs from other organisms can be grouped into these classes, based on amino acid sequence identity and/or gene structure; notable, for example, is ARF3 from *Saccharomyces cerevisiae*, which is a class-III ARF most closely resembling ARF6 (Lee et al. 1994)

β-phosphate binding, whereas region III (NKXD) is required for positioning and specificity of the purine base in the nucleotide site.

2. The Tertiary Structures of ARFs

Before publication of the three dimensional structures of human ARF1–GDP (Amor et al. 1994), rat ARF1–GDP (Greasley et al. 1995), and human $ARF1_t$–GppNHp (a poorly hydrolyzable analogue of GTP; Goldberg 1998), Ras was considered the best available model for ARF structure. When

sequences are compared with the putative GTP-binding and hydrolysis regions aligned, ARF has an N-terminal extension not present in Ras. This leads to the suggestion that this could explain why ARF bound GDP with a higher affinity than it bound GTP and had a strong dependence on phospholipids for nucleotide exchange (KAHN et al. 1992). Structures of ARF1–GDP crystallized as monomers (GREASLEY et al. 1995) and dimers (AMOR et al. 1994; GREASLEY et al. 1995) revealed that ARF differed not only by the addition of an amphipathic α-helix and loop at the N-terminus but also contained an additional β-sheet in the effector region of Ras, through which the molecules in the dimer structure appeared to interact. Other important structural features gleaned from crystallized ARF1–GDP included an apparently positively charged patch on the surface of the molecule, which may be important for its interaction with membrane lipids, and the position of Gln71, which is important for GTP hydrolysis by ARFs (TANIGAWA et al. 1993; TEAL et al. 1994; ZHANG et al. 1994; KAHN et al. 1995). This residue was approximately 5 Å further from the putative γ phosphate-binding site than is Gln61 in the Ras structure. This was mentioned as a possible explanation for the lack of GTPase activity compared with Ras.

Insights into mechanisms of nucleotide release and binding to ARF were gleaned from the crystal structure of human ARF1 lacking the first 17 amino acids ($ARF1_t$, also referred to as Δ 17ARF1; KAHN et al. 1992), with GppNHp bound. As was seen in the ARF1–GDP structures, the core of the activated ARF is mostly composed of β-sheets. The bulk of the differences between ARF–GDP and ARF_t–GppNHp were in residues 38–83, a region described as encompassing switch I, strand β2, loop λ3, strand β3, and switch II, in that order (GOLDBERG 1998). In the inactive or GDP-bound state, residues from loop λ3 form a hydrophobic binding site for one face of the N-terminal amphipathic helix, and switch I forms hydrogen bonds with strand β2. In the activated state, switch I is not hydrogen bonded to β2 but adopts a conformation that allows Thr48 to interact with the Mg^{2+} and the γ-phosphate of the nucleotide. The changes in relationship between switch I and strand β2 are thought to translate to concomitant changes in strand β3, loop λ3, and switch II, resulting in destabilization of the N-terminus by changing the orientation of the amino acids in loop λ3 that form the hydrophobic N-terminal binding pocket and thus making the N-terminus accessible for membrane interaction. Additionally, the changes in strand β3 reorient switch II towards the active site and make it less mobile.

IV. Other ARF Family Members

1. ARF-Related Proteins

ARL proteins are highly identical to ARFs throughout their amino acid sequences, including the consensus regions for guanine-nucleotide binding. Like ARFs, these proteins appear to be ubiquitous in eukaryotes. In many organisms, the presence of both ARF and ARL gene products has been

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     ARL1  MGG----FFSSIFSSLFGT--REMRILILGLDGAGKTTILYRLQVGEVVTTIPTIGFNVE 54
     ARL2  MG------LLTILKKMKQK-ERELRLLMLGLDNAGKTTILKKFNGEDIDTISPTLGFNIK 53
     ARL3  MG------LLSILRKLKSAPDQEVRILLLGLDNAGKTTLLKQLASEDISHITPTQGFNIK 54
  Class II  **       **:**:*:*.  ::*:*:*:**********:**:: .***. *:** *****
     ARL4  MGNGLSDQT-SILSNLPSF--QSFHIVILGLDCAGKTTVLYRLQFNEFVNTVPTKGFNTE 57
     ARL6  MGNHLTEMAPTASSFLPHF--QALHVVVIGLDSAGKTSLLYRLKFKEFVQSVPTKGFNTE 58
     ARL7  MGN---------ISSNISAF--QSLHIVMLGLDSAGKTTVLYRLKFNEFVNTVPTIGFNTE 50
Class III  ***          * :. *  *::*:*::***.****::****:*:***::*** *****

     ARL1  TVTY-----KNLKFQVWDLGGQTSIRPYWRCYYSNTDAVIYVVDSCDRDRIGISKSELVA 109
     ARL2  TLEH-----RGFKLNIWDVGGQKSLRSYWRNYFESTDGLIWVVDSADRQRMQDCQRELQS 108
     ARL3  SVQS-----QGFKLNVWDIGGQRKIRPYWKNYFENTDILIYVIDSADRKRFEETGQELAE 109
  Class II  :::       :*****:**:***:.:*.**:****.** **:*:*****:*:::  :** .
     ARL4  KIKVTLGNSKTVTFHFWDVGGQEKLRPLWKSYTRCTDGIVFVVDSVDVERMEEAKTELHK 117
     ARL6  KIRVPLGGSRGITFQVWDVGGQEKLRPLWRSYNRRTDGLVFVVDAAEAERLEEAKVELFR 118
     ARL7  KIKLSNGTAKGISCHFWDVGGQEKLRPLWKSYSRCTDGIIYVVDSVDVDRLEEAKTELFK 110
Class III  **::. * :: :: :.**************:**.* ***:::***:.:.:*:****.***:

     ARL1  MLEEEELRKAILVVFANKQDMEQAMTSSEMANSLGLPALK-DRKWQIFKTSATKGTGLDE 168
     ARL2  LLVEERLAGATLLIFANKQDLPGALSSNAIREALELDSIR-SHHWCIQGCSAVTGENLLP 167
     ARL3  LLEEEKLSCVPVLIFANKQDLLTAAPASEIAEGLNLHTIR-DRVWQIQSCSALTGEGVQD 168
  Class II  ** **:*: .:********* * .:. * *.*:*.:** .: * **.***:***.:
     ARL4  ITRISENQGVPVLIVANKQDLRNSLSLSEIEKLLAMGELSSSTPWHLQPTCAIIGDGLKE 177
     ARL6  ISRASDNQGVPVLVLANKQDQPGALSAAEVEKRLAVRELAAATLTHVQGCSAVDGLGLQQ 178
     ARL7  VTKFAENQGTPLLVIANKQDLPKSLPVAEIEKQLALHELIPATTYHVQPACAIIGEGLPE 170
Class III  ::: ::***.*:*::*****   :*. :*:** **: ** .:*  *:*  .*: * ** :

     ARL1  AMEWLVETLKSRQ---------- 181
     ARL2  GIDWLLDDISSRIFTAD------ 184
     ARL3  GMNWVCKNVNAKKK--------- 182
  Class II  *::*: .::.::
     ARL4  GLEKLHDMIIKRRKMLRQQKKKR 200
     ARL6  GLERLYEMILKRKKAARGGKKRR 201
     ARL7  GMDKLYEMILKRRKSLKQ-KKKR 192
Class III  *:::*::**:**:*  :  **:*
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Fig. 3. Alignment of human adenosine diphosphate–ribosylation-factor (ARF)-like (hARL)-deduced amino acid sequences. Sequences were aligned using ClustalX for the personal computer (ftp://ftp.ebi.ac.uk/pub/software/dos/clustalw/clustalx). References and accession numbers for GenBank (GB) and SwissProt (SP) databases are: hARL1 (Zhang et al. 1995a) GB-728888 SP-P40616; hARL2 (Clark et al. 1993) GB-2828212 SP-P36404; hARL3 (Cavenagh et al. 1994) GB-543851SP-P36405; hARL4 GB-1168495 SP-P40617; hARL6 (Smith et al. 1995) GB-1351977 SP-P49703; hARL7 GB-3913085 SP-P56559. hARL1 appears to be the single human member of class I, whereas ARLs 2 and 3 and ARLs 4, 6 and 7 constitute classes II and III, respectively. Like ARFs, ARLs from other species may also fall into these groupings

demonstrated. There are six human ARLs that, similar to the human ARFs, appear to fall into classes based on amino acid sequence similarities (Fig. 3). ARLs do not have the ability to rescue the *arf1*$^-$, *arf2*$^-$ double mutant in yeast, stimulate either NAD–$G_{\alpha s}$ or NAD–agmatine ADP-ribosyltransferase activity of CTA1 or enhance PLD activity. Thus, they have generally been thought of as a related, but different class of proteins. Human ARL1 is the only known

protein that appears to "bridge" the ARF and ARL gap. Some CT activation (NAD–agmatine ADP ribosyltransferase activity) was demonstrated in the presence of phosphatidylserine as was some PLD activation in the presence of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol 4,5-bisphosphate (HONG et al. 1998).

ARL1 was first identified in *Drosophila* (TAMKUN et al. 1991). The dARL1 gene, which is essential, codes for a protein that binds and unlike ARFI, hydrolyzes GTP and does not stimulate CT NAD–$G_{\alpha s}$ ADP-ribosyltransferase activity. Golgi localization of ARL1 in rat kidney cells (LOWE et al. 1996), ARL2 in PC-12 cells (ICARD-LIEPKALNS et al. 1997), and ARL1 in yeast (LEE et al. 1997b) was demonstrated by immunofluorescence microscopy. Rat ARL1, upon BFA treatment, redistributed from the Golgi to the cytosol at a rate different from that of ARF or β-COP (coat protein; LOWE et al. 1996). In yeast, ARL1 was not an essential gene; moreover, ARLI was not required for anterograde ER–Golgi transport of carboxypeptidase Y, as shown by its glycosylation pattern (LEE et al. 1997b). Rat ARL2 (also known as ARL184) increased Ca^{2+}-dependent acetylcholine release when overexpressed in PC-12 cells (ICARD-LIEPKALNS et al. 1997). Following ischemia, ARF4L (analogous to ARL6) in gerbil hippocampal neurons was elevated, as were levels of ARF1, ARL4, and β-COP (KATAYAMA et al. 1998).

2. ARF-Domain Protein 1

ARF-domain protein 1 (ARD1) is a 64-kDa protein that contains an 18-kDa C-terminal ARF domain and a 46-kDa N-terminal domain. The putative open reading frame of ARD1 consists of 1722 nucleotides encoding a protein of 574 amino acids. The nucleotide sequence of the ARF domain of ARD1 is 60–66% identical to those of mammalian ARFs. At the amino acid level, the ARF domain of ARD1 is 55–60% identical and 69–72% similar to other ARFs. Human and rat ARD1 are 92% and 98% identical at the nucleotide and amino acid levels, respectively (MISHIMA et al. 1993). Regions common to ARFs that are believed to be involved in guanine-nucleotide binding and GTP hydrolysis are also conserved in ARD1 (MISHIMA et al. 1993). Northern blot analyses revealed 4.2-kb and 3.7-kb mRNAs ubiquitously distributed in rat, mouse, rabbit, and human tissues, suggesting that ARD1 is conserved in eukaryotic cells (MISHIMA et al. 1993). A gene analogous to ARD1, however, was not detected in *Saccharomyces cerevisiae* (Vitale, unpublished observations).

Recombinant proteins were used to demonstrate that ARD1 and its ARF domain specifically bind GDP and GTP in the presence of millimolar concentrations of $MgCl_2$ (VITALE et al. 1997b). As observed for ARFs, certain phospholipids, especially cardiolipin, markedly enhanced binding of GTPγS to ARD1. Accordingly, ARD1 enhanced CTA ADP-ribosyltransferase activity in a GTP- and phospholipid-dependent manner (VITALE et al. 1997b).

The main differences between the ARD1 sequence and those of other ARFs are concentrated in the N- and C-terminal regions of the ARF domain.

In ARD1, the 15 amino acids corresponding in position to the N-terminal α-helix of ARF influence GDP dissociation (Vitale et al. 1997a) in a manner similar to that of the α-helix at the N-terminus of ARF1 (Hong et al. 1994, 1995; Randazzo et al. 1995). Site-specific mutagenesis suggested that hydrophobic residues in this region have a critical effect on GDP dissociation from ARD1 (Vitale et al. 1997a). Because a guanine nucleotide-dissociation inhibitor (GDI) protein for ARFs has yet to be identified, it has been postulated that ARFs and ARD1 have an analogous, intrinsic, regulatory GDI domain (Vitale et al. 1997a).

Although the ARF domain of ARD1 (like ARFs) had no intrinsic GTPase activity, ARD1 hydrolyzed GTP at a significant rate (Vitale et al. 1996). The 46-kDa N-terminal extension, synthesized in *E. coli* separately from the ARF domain, acted as a GTPase-activating protein (or GAP) for the ARF domain of ARD (Vitale et al. 1996). The smallest GTPase-activating domain in ARD1 was contained in the region between amino acids 101 and 333 (Vitale et al. 1998b), which is similar in size to the minimal catalytic domains of the Ras GAPs (Ahmadian et al. 1996). The GAP domain of ARD1 can be divided into a region important for physical association with the ARF domain (residues 200–333) and a smaller region involved in stimulating GTP hydrolysis. Site-specific mutagenesis in the latter region, revealed that a zinc finger, two arginine residues, and a motif that resembles a consensus sequence in Rho/Rac GAPs are required for GAP activity (Vitale et al. 1998b). Mutations that abolished the physical interaction of the GAP and ARF domains of ARD1 also prevented stimulation of GTP hydrolysis, suggesting that a stable association of the two domains is required for GTP hydrolysis (Vitale et al. 1998b).

The GAP activity of the N-terminus of ARD1 is specific for its ARF domain; other ARFs are not substrates (Ding et al. 1996). A small region of seven amino acids in the effector region of the ARF domain is responsible for this specificity (Vitale et al. 1997b). Single or double amino acid substitutions in this segment demonstrated that a proline contributed to the physical interaction with the GAP domain, presumably by creating a curve in the β-sheet structure, which could place important charged residues (Asp427 and Glu428) in the correct position for interaction with the GAP domain (Vitale et al. 1997b). These negatively charged residues are believed to form salt bridges with positively charged Arg249 and Lys250 in the N-terminal GAP region (Vitale et al. 1998b). Similarly, the Ras/GAP association is based on interactions between positively charged residues in GAPs and negatively charged residues in the effector region of Ras (Miao et al. 1996). Hydrophobic interactions have also been postulated to be important for the interaction of the two functional domains of ARD1 (Vitale et al. 1998b).

Native ARD1 was found to be associated with lysosomal and Golgi membranes isolated from human liver by immunoaffinity (Vitale et al. 1998a). Accordingly, when overexpressed in NIH3T3, COS 7, or HeLa cells, ARD1 had a subcellular localization typical of the Golgi apparatus and lysosomes,

which was distinct from that of other ARFs (VITALE et al. 1998a). ARD1, expressed as a green, fluorescent fusion protein, was initially associated with the Golgi network and subsequently appeared in lysosomes, suggesting that ARD1 might undergo vectorial transport between the two organelles (VITALE et al. 1998a). Thus, by analogy to ARFs, ARD1 may participate in transport between the Golgi apparatus and lysosomes.

The covalent attachment of a GAP-like domain to the GTPase core of an ARF protein is unique and presumably critical to regulate the function of ARD1 in vesicular trafficking. Despite the rapid progress made in the molecular characterization of ARD1, which has also helped to reveal some aspects of ARF regulation, the physiological function of ARD1 remains to be established.

V. Molecules that Regulate ARF Function: GEPs and GAPs

1. ARF Guanine Nucleotide-Exchange Proteins

Like other small guanine nucleotide-binding proteins, ARFs cycle between inactive, GDP-bound and active, GTP-bound states (Fig. 4). Phospholipids have been reported to moderately stimulate GTP binding to ARFs and ARD1 (WEISS et al. 1989; TSAI et al. 1994; FRANCO et al. 1995; VITALE et al. 1997b). Although there is still debate whether phosphatidylinositol bisphosphate (PIP_2) can enhance nucleotide release by ARFs in vivo (CHABRE et al. 1998), dissociation of GDP and binding of GTP to ARFs is clearly accelerated by GEPs. ARF GEPs have been purified from cytosolic and membrane-bound fractions and have been classified into two classes according to their size and sensitivity to inhibition by the fungal fatty-acid metabolite BFA. BFA causes disintegration of the Golgi apparatus, presumably by blocking the activation of ARFs by GEPs (DONALDSON et al. 1992b; HELMS and ROTHMAN 1992).

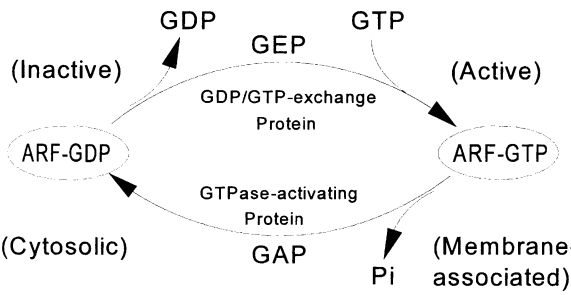

Fig. 4. Model for adenosine diphosphate–ribosylation factor (ARF) activation in cells. ARF guanine nucleotide-exchange proteins (GEPs) and guanosine triphosphatase-activating proteins (GAPs) function to activate and inactivate ARF, respectively. Other factors and molecules that, in turn, regulate GEPs and GAPs (lipids, etc.) are not included in the figure but are discussed in the text

Two ~200-kDa members of a family of large, BFA-sensitive GEPs were purified from bovine brain (MORINAGA et al. 1996). The deduced amino acid sequence of the ~200-kDa protein revealed the presence of a domain with sequences similar to that of the ~230-kDa yeast Sec7 phosphoprotein (MORINAGA et al. 1996, 1997). Sec7 is essential for protein secretion in yeast and moves between membrane and cytosolic fractions (FRANZUSOFF and SCHEKMAN 1989). Interestingly, overexpression of human ARF4 or yeast ARF1/2 rescues Sec7 mutants (DEITZ et al. 1996), consistent with the idea that Sec7 is upstream of ARF, as expected for a GEP. Gea1 and Gea2 are two other yeast proteins with masses of ~160-kDa that contain a Sec7 domain and display ARF GEP activity that is BFA sensitive (PEYROCHE et al. 1996). A series of deletion mutants showed that the Sec7 domain of the ~200-kDa ARF GEP possesses GEP activity (MORINAGA et al. 1999). It was also shown that the yeast Sec7 domain has GEP activity that is sensitive to BFA (SATA et al. 1998).

A family of smaller (~50-kDa) ARF GEPs that are not sensitive to BFA includes two proteins purified from bovine brain and spleen cytosol (TSAI et al. 1994, 1996), and the cytohesin family, which includes cytohesin-1 (MEACCI et al. 1997), cytohesin-2 or ARF nucleotide-binding-site opener (ARNO; CHARDIN et al. 1996), and cytohesin-3 or GRP1 (KLARLUND et al. 1997). In addition to a central Sec7 domain, cytohesins also contain an N-terminal coiled-coil region and a C-terminal pleckstrin homology (PH) domain. PH domains bind negatively charged phospholipids and regulate ARF GEP activity (PARIS et al. 1997). PH domains are also important in GEPs for several Ras family GTPases (HEMMINGS 1997). Specific binding of phosphatidyl nositol 3,4,5-triphosphate to the PH domains of cytohesin-1 and cytohesin-3 suggested that these ARF GEPs may be regulated by signaling pathways involving phosphatidylinositol-3-kinases (KOLANUS et al. 1996).

Recombinant ARNO stimulated GTP binding to myristoylated ARF1 and ARF6 in vitro (FRANK et al. 1998). Expression of ARNO seems to be ubiquitous and localized to the plasma membrane (FRANK et al. 1998). Because the distribution of ARF6 resembles that of ARNO and because the function of ARF6 is also resistant to the action of BFA (RADHAKRISHNA and DONALDSON 1997), it was postulated that ARNO is a BFA-insensitive GEP for ARF6. The function of ARNO as a GEP for ARF6, however, remains controversial (FRANCO et al. 1998; FRANK et al. 1998). In vitro studies performed with recombinant ARNO and myristoylated ARF1 are consistent with the conclusion that the PH domain is not directly involved in catalytic activity but promotes membrane association of ARNO and, therefore, its interaction with membrane-associated ARF (PARIS et al. 1997).

The crystal structure of the Sec7 domain of ARNO contains ten α-helices, the first seven of which are arranged in a right-handed superhelix, with the last three forming a cap at one end (BETZ et al. 1998; CHERFILS et al. 1998; MOSSESSOVA et al. 1998). There is a deep groove on one surface formed by two highly conserved sequences. An area of significant hydrophobicity is located

in the active site in ARNO (Cherfils et al. 1998; Mossessova et al. 1998). Within the active site, a glutamate finger acts to displace Mg^{2+} and the β-phosphate, resulting in destabilization of ARF-bound GDP (Béraud-Dufour et al. 1998; Goldberg 1998). This proposed ARF-GEP mechanism has been compared to those for other GTP-binding proteins (Sprang and Coleman 1998); it is possible that mechanistic differences reflect the particular function of the specific GTP-binding proteins.

The switch-I (residues 41–55) and switch-II (residues 70–80) regions of ARF are protected in the ARNO sec7 domain/(Δ17)ARF1 complex, as seen by protection from hydroxyl-radical cleavage, suggesting that they represent sites of interaction between the two proteins (Mossessova et al. 1998). Interestingly, these regions are analogous in structure and function to other monomeric GTPases (Boguski and McCormick 1993).

The N-terminal ARF helix is critical for stimulation of GTP binding by cytohesin-1 but not by its Sec7 domain (Pacheco-Rodriguez et al. 1998). Cytohesin-1 accelerated GTP binding to a ARF1/3 mixture, ARD1, and yeast ARF3 but not to ARF5, ARF6, and ARLs. The Sec7 domain of cytohesin-1 was less substrate specific. It accelerated GTP binding to ARF5 and ARF6 (Pacheco-Rodriguez et al. 1998). These data indicate that there are elements important for substrate specificity in addition to those in the Sec7 domain of cytohesin-1.

The recognition that Sec7 domains are critical for ARF-GEP activity has been of considerable help in identifying new members of the ARF-GEP family; there is no doubt that more ARF GEPs await discovery. There is evidence that GEPs stabilize the nucleotide-free state of ARF, as has been demonstrated for other GTPases (Boguski and McCormick 1993). The physiological significance of specific ARF–ARF-GEP pairings remains to be established. The signaling pathways that lead to ARF-GEP activation will have to be characterized to understand how extracellular signals affect vesicular trafficking.

2. ARF GTPase-Activating Proteins

Inactivation of ARFs requires hydrolysis of bound GTP. Except for ARD1, which possesses its own covalently associated GAP (Vitale et al. 1996), other members of the ARF family have an extremely low intrinsic GTPase activity and require an additional protein to catalyze GTP hydrolysis. ARF GAP1 and GAP2 were initially isolated from rat liver (Randazzo 1997b). GAP1 corresponds to a ~48-kDa protein that was previously purified (Makler et al. 1995) and cloned (Cukierman et al. 1995), whereas GAP2 is a distinct species. These GAPs have different phospholipid dependencies. PIP_2, phosphatidic acid (PA), and phosphatidyl serine stimulated GAP2 more than GAP1. For these GAPs, substrate specificity is restricted to ARF1–ARF5 (Randazzo 1997b). Activity of GAP1 and GAP2 required the N-terminus of ARF (Randazzo et al. 1994). An apparently distinct ARF GAP of mass ~50 kDa, purified from rat

spleen, had a broader specificity, including ARF6 and ARL1 (DING et al. 1996). For this GAP, the presence of the N-terminal helix of ARF was not strictly required, though activity was reduced in its absence (DING et al. 1996). Accordingly, myristoylation of ARF did not affect the ability of this GAP to accelerate GTP hydrolysis (DING et al. 1996).

GAP1 was recruited to Golgi membranes by oligomerized ERD2 (AOE et al. 1997). This membrane receptor recognizes soluble proteins from the ER that contain a KDEL C-terminal sequence utilized for their retrieval from the Golgi apparatus (LEWIS and PELHAM 1990). GAP1 then inactivates ARF1, producing a phenotype specific for ARF inactivation (AOE et al. 1997). Because diacylglycerols with monounsaturated chains, produced mainly from phosphatidyl-choline (PC) hydrolysis by PLD, dramatically increased the activity of a recombinant fragment of GAP1, it was suggested that PLD activity could be a major regulator of ARF GAP (ANTONNY et al. 1997). Similar effects were observed with Gcs1, an analogous ARF GAP from yeast (POON et al. 1996). It was concluded that the effect of phospholipids on ARF-GAP activity was to increase its concentration at the membrane, where ARF GTP resides. In support of this, PIP_2 was shown to promote the association of GAP with ARF (RANDAZZO 1997a). Thus, GAPs that are activated by PIP_2 or other phosphoinositides are presumably subject to regulation via distinct pathways. In agreement with studies of the GAP domain of ARD1 (VITALE et al. 1998b), truncation and site-specific-mutagenesis experiments on GAP1 demonstrated that a zinc-finger motif is critical for GAP activity (CUKIERMAN et al. 1995). Accordingly, Gcs1 also contains a conserved zinc-finger motif (POON et al. 1996).

Additional ARF GAP family members that appear to link the signaling via ARF to other established signaling pathways have recently been identified by two-hydrid screening. The G protein-coupled receptor kinase-interacting protein GIT1 interacts with β-adrenergic receptor kinase and leads to reduced β-adrenergic receptor internalization through its GAP action on ARF (PREMONT et al. 1998). The phospholipid-dependent ARF GTPase-activating proteins termed ASAPs, described by BROWN et al. (1998), associate with SH3 domains of Src family members and are phosphorylated by them (BROWN et al. 1998), thus possibly linking ARF inactivation to yet another group of signaling pathways.

Based on the crystal structures of Ras GAP (SCHEFFZEK et al. 1996) and Rho GAP (BARRETT et al. 1997) and on results obtained from numerous mutational analyses, it was speculated that an arginine finger is critical for GAP activity. This hypothesis was corroborated by the structural information obtained from crystals of a Ras–Ras GAP complex (SCHEFFZEK et al. 1997). Data from measurements of the activity of the GAP domain of ARD1 also favor the arginine-finger hypothesis (VITALE et al. 1998b), in which arginines stabilize a transition state during GTP hydrolysis (SCHEFFZEK et al. 1997). It is, therefore, very likely that ARF GAPs function in a similar fashion.

VI. Other ARF-Interacting Molecules

1. Phospholipase D

PLD hydrolyzes membrane phospholipids to produce a free head group and phosphatidic acid, a mitogen that can be metabolized further by PA phosphatase to produce the protein kinase C activator, diacylglycerol (Cockcroft 1996; English 1996; Kanaho et al. 1996; Liscovitch 1996; Exton 1997b; Gomez-Cambronero and Keire 1998). Noting that guanine nucleotides and divalent cations (among other cytosolic factors) were required for the stimulation of phosphatidylcholine-specific PLD (Anthes et al. 1991), two groups identified ARF1 (Brown et al. 1993) and ARF3 (Cockcroft et al. 1994) as activators of PC-specific PLD. This ARF-stimulated PLD, later shown to be distinct from that stimulated by oleate (Massenburg et al. 1994), required PIP_2 (Brown et al. 1993; Massenburg et al. 1994; Brown et al. 1995). ARFs from all three classes stimulated this PLD (Massenburg et al. 1994); myristoylation of ARF was not required but did enhance stimulation (Brown et al. 1993; Massenburg et al. 1994).

The first identified (and perhaps the most well studied) ARF-stimulated PLD isoform is human PLD1a (Hammond et al. 1995). PLD1a, which localizes to the ER, Golgi, and late endosomes, was activated by PIP_2 and stimulated by RhoA, Rac, cdc42, and the regulatory domain of protein kinase C (Exton 1997a). Subsequently, PLD1b, product of a shorter, alternatively spliced variant of PLD1a, was shown to have similar characteristics (Hammond et al. 1997). PLD2, which is localized primarily at the plasma membrane (Colley et al. 1997), is 50% identical to PLD1a. Although it had been believed that PLD2 was not stimulated by ARF (Colley et al. 1997; Jenco et al. 1998), activation was recently reported (Lopez et al. 1998).

Elements of ARF structure required for the stimulation of partially purified rat-brain PLD1 were investigated using chimeric proteins composed of ARF and ARL. The N-terminal 73 residues of ARF1 were shown to be necessary and sufficient for PLD1 activation (Zhang et al. 1995a). Further investigation showed that the N-terminal helix was at least one region within the 73 residues contributing to that requirement (unpublished data).

The proposed functions of ARF-dependent PLD include the regulation of specific intracellular signaling events and the regulation of vesicle formation (as discussed below). Several additional potential regulators or interacting proteins that may provide insight into the roles of PLD isoforms in cells have been described. The stimulation of PLD by its reaction product (PA; Geng et al. 1998), along with the known stimulation by PIP_2, may be consistent with a role for PLD in the regulation and production of negatively charged phospholipids. Several groups have identified PLD1 inhibitors that specifically interact with (Kim et al. 1996; Lukowski et al. 1998) or hydrolyze (Han et al. 1996; Chung et al. 1997) PIP_2. Regulators that act independently of PIP_2 are the clathrin assembly protein AP3, which inhibits PLD1 (Lee et al. 1997a), and the synucleins, which inhibit PLD2 (Jenco et al. 1998). More-

over, the association of RalA with PLD1 in vitro did not result in PLD stimulation (Luo et al. 1998) but, in cells, might provide a link to Ras signaling pathways.

2. Arfaptins

Arfaptins 1 and 2 were identified as proteins that interact with ARF3(Q71L), a constitutively active, GTP-bound form, in a yeast two-hybrid screen of an HL60 complementary DNA library (Kanoh et al. 1997). In vitro, these proteins preferentially bound to class-I ARFs, especially ARF1, but only in the GTP-bound active state. Arfaptin 1 was recruited to Golgi membranes in an ARF-dependent and BFA-inhibited manner. Insight into arfaptin function came from subsequent studies, in which arfaptin 1 inhibited activation of both CT and PLD in vitro (Tsai et al. 1998). ARF mutants in which the N-terminus was lacking or was replaced by the corresponding ARL sequence were not affected by arfaptin 1. PLD activation by the mutant ARF that contained only the N-terminal 73 amino acids of ARF was inhibited by arfaptin 1. Taken together, these observations are consistent with the idea that arfaptin interaction involves the N-terminus of ARF. ARF–arfaptin interaction could be a mechanism for regulation of intracellular levels of active ARF.

VII. ARF in Cells

1. ARFs' Role in Vesicular Trafficking Events

Disruption of the ARF1 gene in yeast first suggested a cellular role for ARF as a regulator of vesicular trafficking events. Stearns and coworkers (1990b) observed that invertase secretion was defective and that incompletely glycosylated invertase accumulated inside of *arf1*$^-$ yeast (*S. cerevisiae*). Disruption of both yARF1 and yARF2 genes was lethal (Stearns et al. 1990a), but disruption of the yARF3 gene was not (Lee et al. 1994). These observations are consistent with roles for yARFs 1 and 2 (but not yARF3) in secretion. Subsequently, ARF was shown to be required for an early step in ER-to-Golgi transport (Balch et al. 1992), and it was associated with vesicular COPs on the surface of non-clathrin-coated, Golgi-derived vesicles (Serafini et al. 1991). Furthermore, GTP-dependent activation of ARF facilitated the binding of β-COP to Golgi-derived vesicles (Donaldson et al. 1992a; Palmer et al. 1993) via a process that was shown to be inhibited by BFA (Donaldson et al. 1992b). ARF and β-COP, which is one of seven COPs that constitute the COPI protein complex, function in ER and Golgi transport (Rothman 1996). Promotion of ARF-dependent β-COP (and thus COPI) binding to membranes by AlF_4 suggested a role for heterotrimeric G proteins in the regulation of COPI-coated vesicle formation (Donaldson et al. 1991; Finazzi et al. 1994). ARF also mediates the binding of both clathrin adaptor protein (AP)-1 (Stamnes and Rothman 1993; Traub et al. 1993; Dittié et al. 1996; Zhu et al. 1998) and AP-3 (Ooi et al. 1998) to membranes. In contrast, the Sar proteins (but not

ARF) control the formation of and are associated with COPII-coated vesicles that function in anterograde transport from the ER (KUEHN and SCHEKMAN 1997; BARLOWE 1998). COPI was shown to be involved in anterograde and retrograde vesicular traffic between the ER and Golgi and within the Golgi, although there is a great deal of controversy concerning the exact roles of COPI vs. COPII protein coats (GAYNOR et al. 1998).

In addition to its apparently direct effect on vesicular traffic by recruitment of the appropriate protein coat to initiate vesicle budding, ARF may also modify the physical properties of the membrane to facilitate vesicle formation through the activation of PLD. The addition of an exogenous bacterial PLD to Golgi membranes (without the addition of ARF) stimulated the binding of COPI (KTISTAKIS et al. 1996), consistent with a role for PA in coat recruitment and vesicle formation. In addition, it was reported that COPI-coated vesicles could be formed from liposomes that contained PA, without the addition of exogenous ARF (KTISTAKIS et al. 1996). In support of a role for PLD, primary alcohols, which cause formation of the transphosphatidylation product phosphatidylethanol instead of PA, inhibited the transport of viral glycoproteins from ER to the Golgi (BI et al. 1997). Subsequent reports indicate an absolute requirement for ARF in COPI-vesicle formation not merely a catalytic role, as might be inferred if PLD activation were sufficient for vesicle formation (SPANG et al. 1998; STAMNES et al. 1998). The finding that significant changes in the total PA content of Golgi membranes were not detected during COPI-dependent budding (STAMNES et al. 1998), coupled with the observation that yeast PLDs were neither activated by ARF nor required for growth (RUDGE et al. 1998) leaves the roles of PLD and PA in COPI-vesicle dynamics unestablished.

2. Subcellular Localization of ARF

Not surprisingly, it is becoming increasingly apparent that ARF class distinctions, originally made entirely on the basis of structural information (protein, gene), are related to specific intracellular localization and possibly function. Yeast ARF1 was the first to be localized to the Golgi (STEARNS et al. 1990b). Based on studies with recombinant wild-type and mutant human and native bovine ARFs, this localization appears to be a characteristic of all ARFs that could be grouped as ARF class-I members (TSAI et al. 1992; TANIGAWA et al. 1993; TEAL et al. 1994, ZHANG et al. 1994; KAHN et al. 1995). Although endogenous ARF5 (class II) was reported to bind to Golgi membranes (TSAI et al. 1992, 1993; HAUN et al. 1993), information on the function and subcellular distribution of ARFs 4 and 5 is conspicuously lacking. Most distinct in its localization is the class-III ARF human ARF6, which (when overexpressed) was localized and appeared to function at the plasma membrane (D'SOUZA-SCHOREY et al. 1995; PETERS et al. 1995; RADHAKRISHNA et al. 1996). Native ARF6 in a variety of cells was later shown to predominantly localize at the plasma membrane (CAVENAGH et al. 1996; YANG et al. 1998), although some

staining of both the cytosol and other intracellular membranes was noted (YANG et al. 1998). In adrenal chromaffin cells, native ARF6 was found to be associated with secretory granules (GALAS et al. 1997) and redistributed to the plasma membrane upon cell stimulation (CAUMONT et al. 1998).

D. Summary

CT ADP-ribosylates an arginine within $G_{\alpha s}$, leading to an increase in intracellular cAMP concentration. It is not known whether CT encounters ARF proteins within the cell during intoxication, but the in vitro stimulation of CT by ARFs has provided an invaluable tool for the study of their function. As well-conserved molecules distinct to eukaryotes, ARFs are now known to function in events and interact with molecules, all of which appear to have roles in vesicular trafficking. The intricacy with which ARFs are regulated by protein and lipid cofactors, and the presence of several ARFs within a single cell, lead to the postulation that their role in cells is more than simply regulation of bulk vesicular transport. It will be important to determine what signals lie upstream of ARF activation and whether these signals have some vesicular transport component as a common theme.

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CHAPTER 8

Pertussis Toxin: Structure–Function Relationship

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A. Introduction

Pertussis toxin (PTX), produced and secreted by *Bordetella pertussis*, the etiologic agent of whooping cough, is the most complex bacterial toxin known today. It is a multimeric protein with a total molecular mass of 105 kDa and is composed of five dissimilar subunits (Fig. 1). The PTX subunits are named S1–S5 according to their decreasing molecular weights (TAMURA et al. 1982). They range in size from 11 kDa for the S5 subunit to 26 kDa for S1 (LOCHT and KEITH 1986; NICOSIA et al. 1986). The subunits are arranged in a hexameric structure in which each subunit is represented in a single copy except for S4, which is present in two copies for each toxin molecule.

Functionally, PTX can be subdivided into two major moieties (Fig. 1). The A protomer, corresponding to the S1 subunit, expresses enzymatic ADP–ribosyltransferase activity, and the B oligomer, composed of subunits S2–S5, is responsible for the binding of PTX to its target-cell receptors. This toxin is, therefore, a member of the A-B toxin family.

The crystal structure of PTX has been solved at a 2.9-Å resolution (STEIN et al. 1994a). The toxin has the shape of a pyramid with a triangular base formed by the B-oligomer subunits. The S1 subunit rests on the top of the pyramid. The center of the pyramid consists of a barrel composed of five α-helices, each one belonging to a different B-oligomer subunit. The C-terminus of the S1 subunit penetrates halfway into the pore of the barrel (Fig. 1). The barrel is surrounded by 30 antiparallel β-strands forming a five-fold symmetry that corresponds to five similar folds of approximately 100 residues of each of the B subunits. However, whereas the S4 and S5 subunits contain 110 and 99 residues, respectively, S2 and S3 each contain 199 residues (LOCHT and KEITH 1986; NICOSIA et al. 1986). Compared with S4 and S5, the latter two subunits contain an N-terminal extension which protrudes out of the pentameric domain. S2 and S3 share approximately 70% amino acid identity.

The fold of the 175 N-terminal residues of S1 is similar to that of the enzymatic portion of cholera toxin and of the *Escherichia coli* heat-labile toxin, both of which share catalytic properties similar to those of PTX. Residues 176–235 have no structural homology to the other two toxins, as this region is involved in the interaction with the B oligomer. In addition to the half-way penetration of the C-terminus of S1 into the B-oligomer pore, S1 forms many

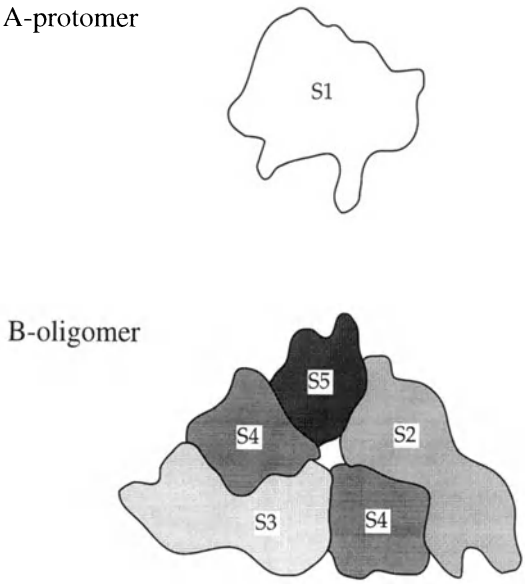

Fig. 1. Schematic structure of pertussis toxin (PTX). The A protomer (*top*), composed of the S1 subunit, rests on top of the B oligomer (*bottom*), composed of subunits S2–S5, by the half-way insertion of its C-terminal end in the center of the triangular base of the pyramidal structure of PTX

contact points with the B moiety, resulting in a tight association that depends on the intramolecular disulfide bond of S1 (Antoine and Locht 1990).

Two disulfide bonds are conserved in all the B subunits. S2 and S3 each contain an additional N-proximal disulfide bond. The associations between adjacent B-oligomer subunits involve antiparallel β-sheets. The strongest associations are those of S2 with S4 and of S3 with S4. The dissociation of these heterodimers requires 8 M urea, whereas incubation of PTX in 5-M urea results in the dissociation of the toxin into the S1 and S5 monomers and the S2/S4 and S3/S4 dimers (Tamura et al. 1982). The structure of the approximately 100 N-terminal extensions of S2 and S3 resembles somewhat the fold of the lectin domain of the mannose-binding protein. One of the two disulfide bonds of the mannose-binding protein is conserved in the S2 and S3 subunits.

B. The Receptor-Binding Activity of PTX

Receptor binding of PTX is mediated by the B oligomer. Although precise receptors have not been identified yet, the toxin can bind to glycoproteins, such as haptoglobin and fetuin, and is also able to agglutinate erythrocytes via its B oligomer. Many cells appear to display PTX receptors on their surfaces, but these seem to vary from cell type to cell type. The receptors are generally

believed to be glycoproteins, but PTX can also bind to the glycolipid G_{D1a} with high affinity (HAUSMAN and BURNS 1993).

The glycoprotein receptors vary in size from roughly 43 kDa on the surface of human T lymphocytes (ROGERS et al. 1990) to over 200 kDa on pancreatic β cell-derived insulin-secreting cells (EL BAYA et al. 1995). Their sugar moieties play an important role in PTX recognition. Treatment with sialidase abolishes PTX binding, and cell lines that lack the carbohydrate sequence NeuAcα(2,6)-Galβ4GlcNAc on glycoproteins become resistant to the toxin (BRENNAN et al. 1988). Therefore, the terminal sialic acid and at least part of the Galβ4GlcNAc sequence may well contribute to the receptor structure of PTX. However, the sugar sequence alone is not sufficient for PTX binding, since different glycoproteins containing the same sugar moieties are differentially recognized by the toxin (ARMSTRONG et al. 1988).

The approximately 100 residues N-terminal domains of the S2 and S3 subunits are structurally similar to a family of calcium-dependent (C-type) lectins, although they lack the functional region (STEIN et al. 1994a). However, the C-terminal domains of these subunits adopt a similar fold as the B subunits of other carbohydrate-binding toxins, suggesting perhaps that the subunits may contain more than one carbohydrate-recognition site.

An undecasaccharide has been co-crystalized with PTX, and the structure of the complex has been solved at a 3.5-Å resolution (STEIN et al. 1994b). This carbohydrate is bound to both S2 and S3 at equivalent sites. The terminal sialic-acid–galactose moieties of the undecasaccharides were found in a similar conformation in both binding sites. The galactose does not interact directly with the protein, but the sialic acid is within hydrogen-bonding distance to the polar and charged groups of Tyr-102, Ser-104 and Arg-125, respectively. The carboxylate group of the sialic acid is in hydrogen-bond contact with Ser-104, and the sugar ring of the sialic acid makes hydrophobic contacts with the aromatic rings of Tyr-102 and Tyr-103. These residues are conserved between S2 and S3.

However, differences in relative binding affinities of the S2/S4 and the S3/S4 dimers have been described (LOBET et al. 1993) with respect to different PTX receptors or model glycoproteins, indicating the existence of several classes of PTX receptor. They may vary in both their polypeptide and carbohydrate portions. When either Tyr-102 and Tyr-103 or Asn-105 of S2 are deleted or replaced (LOBET et al. 1993; LOOSMORE et al. 1993), the toxin loses its ability to bind to haptoglobin or fetuin. Conversely, when either Lys-105 or Tyr-102 and Tyr-103 of S3 are deleted, it fails to interact strongly with its receptor on the surface of Chinese hamster ovary (CHO) cells. In addition, it has been known for some time that iodination of PTX severely reduces its biological activities, suggesting that tyrosine residues are important for these activities (ARMSTRONG and PEPPLER 1987). Fetuin coupled to agarose can prevent inactivation of the toxin upon iodination, suggesting that iodination affects the receptor-binding activity of the toxin. The deletion of other residues in S3 located upstream of the sialic acid binding site, such as Lys-10 or Lys-93, also

affects toxin activity in CHO cells, indicating that the N-terminal domain of this subunit may also be involved in receptor recognition. This domain appears to direct some specificity of PTX-binding to distinct target cells. The N-terminal domain of recombinant S2 has been shown to reconstitute adherence of toxin-deficient *B. pertussis* strains to ciliated cells, whereas that of recombinant S3 reconstitutes binding to macrophages (Saukkonen et al. 1992). Since this domain has a C-type lectin fold but lacks the long carbohydrate-binding loop that forms the functional portion of the lectin fold, it may perhaps recognize regions distal from the sialylated sugar moieties on PTX receptors.

One of the biological activities expressed by the PTX B oligomer alone is mitogenicity for T lymphocytes. The deletions of Asn-105 in S2 and of Lys-105 in S3 abolish the mitogenic activity of PTX for T cells from mouse spleen (Lobet et al. 1993). However, alterations of these residues did not affect the mitogenicity for human lymphocytes, which was instead significantly diminished by changes of Tyr-82 of S3 (Loosemore et al. 1993). This difference may reflect structural differences between the PTX receptors on human and murine T lymphocytes.

Interestingly, PTX is also able to bind to lipopolysaccharide (LPS) via an interaction that specifically involves its S2 subunit but not S3 (Lei and Morrison 1992). The functional role of this interaction has not been demonstrated yet, but it is intriguing to note that alterations of certain amino acids of S2 through site-directed mutagenesis, such as Tyr-102 or Tyr-103, result in poor toxin secretion by *B. pertussis* (Loosemore et al. 1993), whereas alterations of the conserved residues in S3 do not appear to alter toxin production or secretion. Since these residues are directly involved in the carbohydrate-binding activity of S2, it is possible that they also contribute to the binding of LPS, and that the S2-LPS interaction plays a role in some step of toxin secretion. Preincubation or coincubation of PTX with fetuin abrogates the ability of LPS to bind to S2. The lipid-A moiety of LPS is also able to inhibit the S2–LPS interaction (Lei and Morrison 1993), suggesting that S2 may directly bind the lipid-A component of the LPS molecule. The sugar portion of the lipid-A may have some resemblance to part of the carbohydrate moieties of PTX receptors (Fig. 2).

C. Membrane Translocation of PTX

Although some activities of PTX, such as mitogenicity, haemagglutination and binding to leukocytes and ciliated epithelial cells, are directly mediated by the B oligomer of the toxin, most biological activities require, in addition, membrane translocation and the expression of the enzymatic activity of the S1 subunit subsequent to binding of the toxin via its B oligomer. The translocation step occurs after the toxin has been incorporated into intracellular vesicles by receptor-mediated endocytosis and routed via a retrograde transport system involving the Golgi complex and possibly the endoplasmic reticulum.

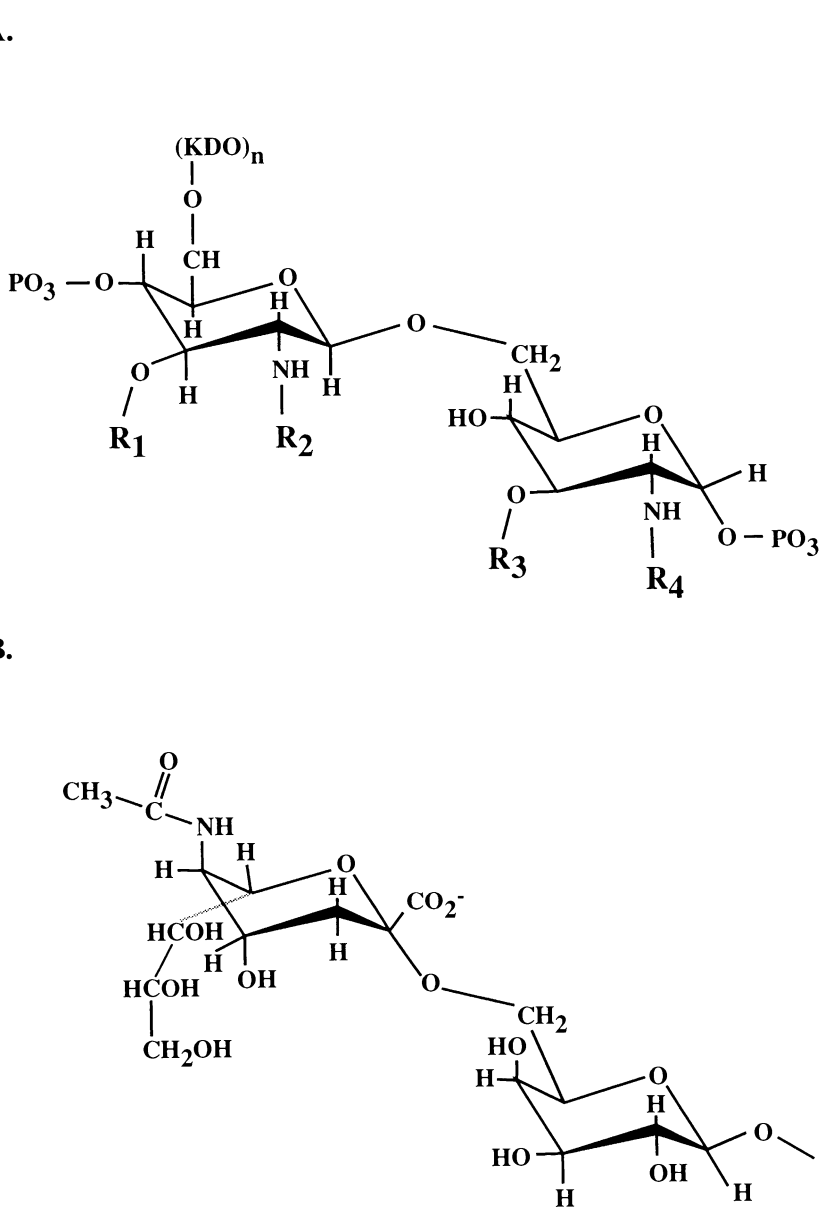

Fig. 2. Comparison of the lipid A portion of lipopolysaccharide (LPS) with the distal saccharides of pertussis-toxin (PTX) receptors. A typical lipid A portion of LPS from enterobacteria (A) contains a disaccharide to which fatty-acid side chains are attached (R1–R4). Lipid A is linked to 2-keto-3-deoxy-D-mannooctonic acid (*KDO*). The terminal chain of the PTX-receptor carbohydrate moiety that binds to the receptor-binding site of the B oligomer, as determined by crystal-structure analysis (STEIN et al. 1994b), contains a disaccharide composed of sialic acid and galactose linked by an $\alpha(2,6)$ bond (B)

Brefeldin A, which causes disassembly of the Golgi apparatus, protects CHO cells (Xu and Barbieri 1995) and insulin-secreting pancreatic cells (El Baya et al. 1997) from PTX action. In cell lines, such as MDCK cells, in which the Golgi apparatus is not affected by Brefeldin A, the drug does not inhibit PTX action, consistent with the involvement of the Golgi in PTX trafficking. Mutant CHO cells that contain a Golgi apparatus whose structure is temperature sensitive are resistant to PTX at non-permissive temperatures (El Baya et al. 1997), again supporting the involvement of an essential Golgi step. In addition, unlike the case for diphtheria toxin, translocation of PTX does not strictly depend on a pH gradient (Hausman and Burns 1992), arguing against translocation at an early, acidified endosomal compartment. However, the addition of NH_4Cl (Xu and Barbieri 1995) or of chloroquine (Xu and Barbieri 1996) to CHO cells inhibited PTX action, suggesting that a pH-sensitive step is involved in toxin trafficking. Electron microscopy and subcellular fractionation studies of PTX-treated CHO cells have confirmed that PTX enters cells via coated pits and then reaches the endosomal compartment and the Golgi complex (El Baya et al. 1997). The partial inhibition of PTX trafficking by microtubule-disrupting agents, such as nocodazole (Xu and Barbieri 1996), suggests that the toxin transits via both a nocodazole-sensitive and a nocodazole-insensitive pathway, consistent with the notion of receptor-mediated endocytosis and at least partial trafficking through late endosomes. However, much of the toxin is routed for degradation towards the lysosomal compartment, similar to other toxins (Johannes and Goud 1998). Xu and Barbieri (1995) proposed a model in which the toxin travels back to the cell surface. However, in contrast to some other bacterial toxins, such as Shiga toxin, for which a full retrograde transport has clearly been demonstrated in a number of ways (Johannes and Goud 1998), relatively little information is available on the intracellular trafficking of PTX.

In addition, where exactly the membrane translocation step of the S1 subunit occurs is not known. Xu and Barbieri (1995) proposed that S1 remains membrane associated even after translocation, because only membrane-bound PTX-substrate proteins are ADP–ribosylated in vivo, but not soluble protein substrates, which are present in postnuclear supernatant fractions of CHO cell extracts. Whether the translocation step requires the help of the B oligomer has also not been clearly established yet, but several observations suggest that the translocation step *sensu stricto* may be independent of the B oligomer. PTX can bind to model membranes consisting of phosphatidylcholine even in the absence of functional glycoconjugate receptors (Hausman and Burns 1992). However, this binding is poor unless adenosine triphosphate (ATP) and reducing agents are added. In the presence of both ATP and reducing agents, the S1 subunit binds efficiently to the model membranes, whereas the binding of the B oligomer remains low. Isolated S1 avidly binds to membranes even in the absence of ATP as long as reducing agents are added, indicating that ATP essentially serves to release S1 from the B oligomer and suggesting that the S1 subunit (in a reduced state) may directly interact with lipid membranes

without the help of the B oligomer. Cleavage of the C-terminal 40–60 amino acid residues of S1 abolishes binding of this subunit to the membranes. This region corresponds to the major hydrophobic domain of the subunit (Antoine and Locht 1990), suggesting that the hydrophobic C-terminal portion of S1 may be the translocation determinant.

The enhancement of S1-mediated toxin binding to membranes by ATP does not appear to require the hydrolysis of ATP, since ADP (Burns and Manclark 1986) or non-hydrolyzable ATP analogues, such as App(NH)p, also stimulate toxin activities (Moss et al. 1986). Instead, ATP may fulfill some structural requirement for toxin activation. Activation of PTX by ATP depends on the presence of the B oligomer (Burns and Manclark 1986), and ATP has been shown to directly bind to the isolated B oligomer and to the holotoxin molecule, albeit in somewhat different buffer conditions (Hausman et al. 1990). Competition experiments have indicated that ATP binding to the B oligomer involves a site different from the receptor-binding site, although fetuin was found to partially interfere, perhaps sterically, with ATP binding.

Recently, the crystal structure of the PTX–ATP complex has been determined; it confirmed that the ATP-binding site is located in the B oligomer (Hazes et al. 1996). It was clearly shown that ATP binds stoichiometrically within the B-oligomer pore and has extensive van der Waals interactions with the protein. The adenine ring is deeply buried and packed between the α helices of the S5 subunit and one of the S4 subunits. The ribose moiety is also buried, and its 2′ and 3′ hydroxyl groups make hydrogen bonds with the side chains of two amino acids of one of the S4 subunits. The phosphate groups are partially exposed and hydrogen bonded to several positively charged amino acids of the S2, S3 and S4 subunits. The negative charges of the triphosphate moiety of ATP displace the C-terminal, negatively charged residues of the S1 subunit due to electrostatic repulsion and steric hindrance. This repulsion, caused by the entering of ATP into the B-oligomer pore from the side opposite the S1 subunit may cause a destabilization of the S1–B oligomer interactions and thus promote the release of the S1 subunit from the B oligomer.

Since ATP is usually found in the cytosol of eukaryotic cells, the ATP-mediated release of S1 necessary for membrane translocation raises a question about the physiological significance of ATP binding in vivo. In addition to the cytosol, the only intracellular compartment known to contain ATP is the endoplasmic reticulum (Braakman et al. 1992), which also contains protein disulfide-isomerases that may help reduce the intramolecular disulfide bond of S1. This suggests that toxin activation and membrane translocation occur within this subcellular compartment.

An attractive model for membrane translocation of PTX integrating all the lines of evidence available has been proposed by Hazes and Read (1997). After trafficking through the Golgi network, the toxin may reach the endoplasmic reticulum, where the presence of ATP could constitute the signal for

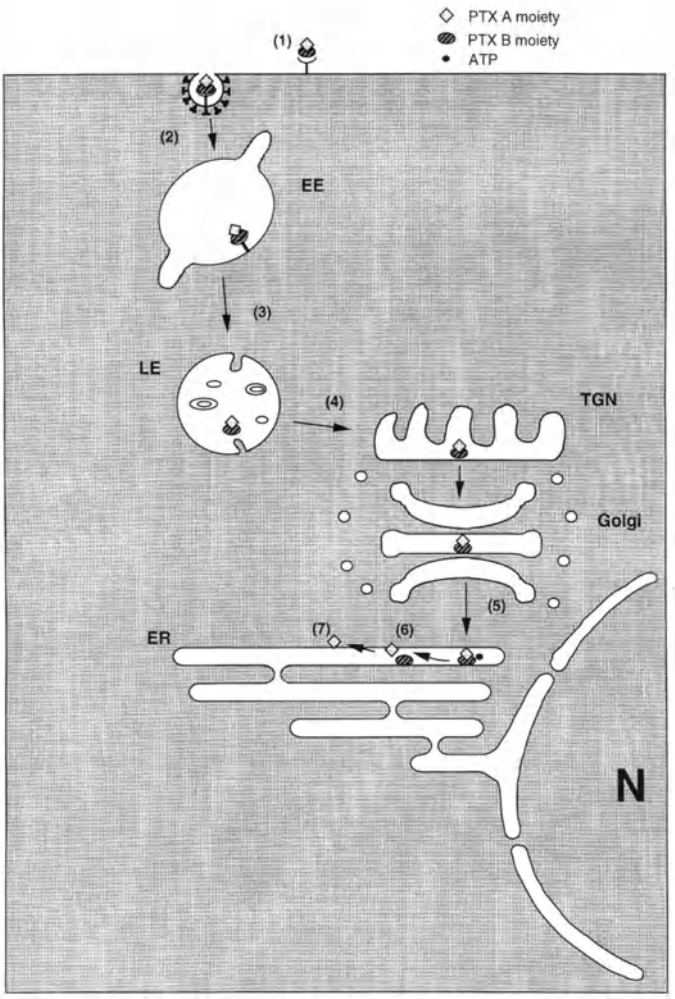

Fig. 3. Model for intracellular trafficking and membrane translocation of pertussis toxin (PTX). In a first step, PTX binds to a cell-surface receptor (*1*) and is internalized by receptor-mediated endocytosis (*2*). The toxin is then transferred to early (*EE*) and late (*LE*) endosomes (*3*) and reaches the trans-Golgi network (*TGN*; *4*). It may undergo an unconventional retrograde transport across the Golgi before reaching the endoplasmic reticulum (*ER*; *5*). In this compartment, the A protomer may dissociate from the B oligomer under the effect of the presence of adenosine triphosphate (*6*). The A protomer can then translocate across the membrane, possibly without the help of the B oligomer (*7*). *N* nucleus

triggering the dissociation of S1 from the B oligomer. Within this compartment, S1 may translocate into the cytosol (Fig. 3) either by its ability to directly interact with lipid membranes or by the use of a retrotranslocation machinery that is employed in a normal cellular process (known as ubiquitin-mediated, endoplasmic-reticulum-associated degradation) for the removal

of improperly folded proteins. However, instead of being degraded in a ubiquitin-dependent fashion by this process, the S1 subunit may simply cross the membrane to exert its effect on the cytosolic site. Hazes and Read (1997) proposed that S1 is protected from ubiquitin-dependent proteolytic degradation because of the absence of lysine in this subunit, since ubiquitination occurs at lysine residues.

Other working hypotheses cannot, of course, be ruled out. Unlike *Pseudomonas aeruginosa* exotoxin A or cholera toxin, which contain clearly identified endoplasmic-reticulum retention signals in the form of the amino acid sequence KDEL (Lord and Roberts 1998), PTX does not contain such sequences. However, PTX may interact with undefined cellular protein intermediates that may interact with KDEL receptors in a more complex fashion. Alternatively, other signals not yet identified in PTX may be involved in endoplasmic-reticulum routing.

D. The Enzymatic Activities of S1

Once internalized into the cytosol, the S1 subunit can catalyze the transfer of the ADP–ribose moiety of nicotinamide adenine dinucleotide $(NAD)^+$ onto an acceptor substrate protein in a reaction called the ADP–ribosyltransferase reaction. As such, PTX is a member of the ADP–ribosylating toxin family, which includes diphtheria toxin, cholera toxin, *E. coli* heat-labile toxin, *P. aeruginosa* exotoxin A and exoenzyme S, and many others that constitute key virulence factors for their respective producing micro-organisms. All these toxins have in common the use of NAD^+ as the donor substrate for the ADP–ribose moiety. They differ in the specificity of their protein acceptor substrate; although the acceptor substrates generally are all guanosine triphosphate (GTP)-binding proteins, their cellular functions are usually distinct. The physiologically relevant acceptor substrate for PTX is the α subunit of the Gi/Go protein family, although water can also serve as an ADP–ribosyl acceptor, albeit with much less efficiency. This latter reaction is termed the NAD^+–glycohydrolase reaction, and its physiological significance is not known.

The Gi and Go proteins are members of the signal-transducing, GTP-binding proteins. Gi serves as a relay from the hormone–receptor complex to the adenylate-cyclase effector enzyme and is destined to down-regulate the adenylate cyclase activity in response to a receptor-mediated extracellular signal. G proteins are usually composed of three subunits: the α, β and γ subunits. The α subunit is able to bind GTP, which activates the protein to transduce the signal to the effector protein which, in the case of the Giα, results in an inhibition of adenylate-cyclase activity. Binding of GTP to the α subunit is triggered by the activation of the cognate receptor. Intrinsic GTPase activity of the Giα subunit results in the hydrolysis of GTP to guanosine diphosphate and inactivates the system. The ADP–ribosylation of Giα uncouples the G

protein from its cognate receptor, and signals that normally should inhibit adenylate cyclase activity are no longer able to do so. As a consequence, the cyclase activity is constitutively de-repressed, and the intracellular levels of cyclic adenosine monophosphate (cAMP) are artificially elevated (Locht and Antoine 1997). Since cAMP is an important secondary messenger involved in the regulation of many important metabolic processes, abnormal intracellular concentrations of cAMP resulting from PTX intoxication have many different physiological effects. It has long been recognized that PTX exerts a wide diversity of biological activities, including leucocytosis, stimulation of insulin secretion, sensitization to histamine, changes in vascular permeability, stimulation of lipolysis in adipocytes, inhibition of macrophage migration, and immunomodulation (Munoz 1985). This diversity explains the different names originally given to PTX, such as lymphocytosis-promoting factor, islet-activating protein and histamine-sensitizing factor.

E. The Enzyme Mechanism of S1-Catalyzed ADP–Ribosylation

The amino acid residue that serves as the ADP–ribose acceptor is a cysteine (Hsia et al. 1985) located four residues from the C-terminal end of the Gα subunit. In fact, all trimeric G proteins tested so far that contain a cysteine residue at this position serve as PTX substrates. Isolated α subunits are poor substrates, and the presence of both the β and γ subunits is required for significant ADP–ribosylation. However, a synthetic peptide corresponding to the last 20 residues of Giα has been shown to serve as an acceptor substrate for PTX (Graf et al. 1992).

Cysteines are unique substrates for bacterial-toxin-catalyzed ADP–ribosylation. Although free cysteine has been reported to serve as an ADP–ribosyl acceptor substrate (Lobban and Van Heyningen 1988), the product of this reaction is different from ADP–ribosylcysteine in the G proteins formed by PTX in the presence of NAD^+ (McDonald et al. 1992). Whereas the use of free cysteine results in the formation of ADP–ribosylthiazolidine, the physiological ADP–ribosylcysteine product in PTX-modified proteins contains a thioglycosidic bond. It is likely that ADP-ribosylthiazolidine is formed through a non-enzymatic reaction of free cysteine with ADP–ribose, which results from the cleavage of NAD^+ catalyzed by PTX in the NAD^+–glycohydrolase reaction. The physiological thioglycosidic bond involves the cysteine sulfhydryl group and the ribose anomeric carbon. The reaction product has an α configuration at the ribose ring, whereas the NAD^+ substrate has a β configuration (Scheuring and Schramm 1995). The PTX-catalyzed ADP ribosylation thus occurs with inversion of the configuration at the 1′ carbon of the ribose ring (Fig. 4), similar to what has been found for the reaction catalyzed by diphtheria and cholera toxins. The inversion of the configuration suggests that the attacking nucleophilic acceptor substrate may

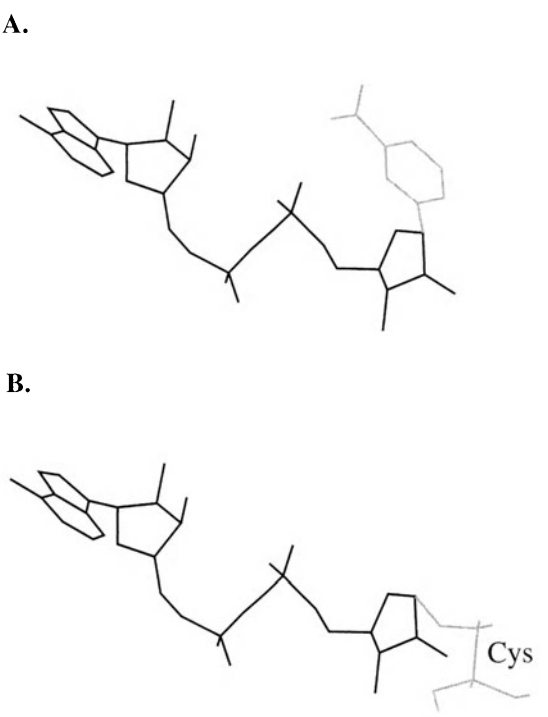

Fig. 4. Comparison of the structure of β-nicotinamide adenine dinucleotide $(NAD)^+$ and the α-adduct on cysteine. The structure of β-NAD^+ has been determined within the active site of diphtheria toxin (A) by Bell and Eisenberg (1996) and is compared with the proposed structure for the adenosine diphosphate (ADP)–ribosylation product of cysteine catalyzed by the S1 subunit of pertussis toxin in an α configuration (B). The ADP–ribose moiety of NAD^+ or ADP–ribose–cysteine is represented in *black*, and the nicotinamide (A) and cysteine (B) portions are in *gray*

transiently associate with the leaving nicotinamide group on the C1 carbon of the ribose during the reaction. This is suggestive of an S_N2-type reaction mechanism involving an oxocarbenium intermediate (Locht and Antoine 1995).

Kinetic-isotope-effect characterization of the transition-state intermediate during NAD^+ glycohydrolysis catalyzed by PTX confirmed that it does indeed have oxocarbenium-ion character (Fig. 5) in the ribose, with significant bond order to the nicotinamide ring corresponding to a distance of 2.14 Å (Scheuring and Schramm 1997a). In this reaction, water is a weak nucleophile, with the water oxygen no closer than 3.5 Å from the reaction center. The nicotinamide–ribose *N*-glycosidic bond length in the transition-state structure was slightly reduced to 2.06 Å when the isotope effects were measured in the ADP–ribosyltransferase reaction using the 20-residue-long C-terminal Giα peptide (Scheuring and Schramm 1997b). In that case, the incoming thiolate

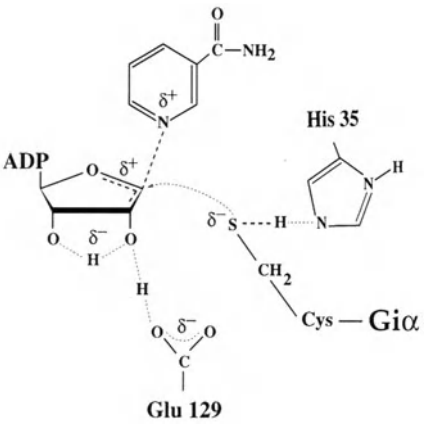

Fig. 5. Proposed enzyme mechanism of S1-catalyzed adenosine diphosphate ribosylation. The oxocarbenium-ion character of the transition-state intermediate is promoted by the interaction of the carboxylate group of Glu-129 with the 2′ OH group of the ribose. This weakens the *N*-glycosidic bond, which is subjected to a nucleophilic attack by the sulfur of the acceptor cysteine activated by His-35 via hydrogen bonding or even ionization (adapted from Locht and Antoine 1997)

has a bond order that corresponds to a 2.47-Å distance, which provides an increased S_N2 character in a loose transition state, with a strong oxocarbenium character in the ribose. The kinetic isotope effects observed are consistent with deprotonation of the attacking cysteine prior to transition-state formation (Fig. 5). The sulfur of the cysteine in the G protein appears to participate more actively as an attacking nucleophile in the ADP–ribosyltransferase reaction than does the oxygen of the water molecule in the NAD^+–glycohydrolyze reaction. The catalytic rate of the PTX-catalyzed ADP–ribosyltransferase reaction is consistently about one order of magnitude higher than that of the NAD^+–glycohydrolyze reaction (Cortina et al. 1991; Antoine et al. 1993).

The oxocarbenium intermediate is stabilized by its positive charge created by the delocalization between the C1′–O4′ and C1′–C2′ bonds, with the total bond order at C1′ held constant. The thiolate anion of the attacking cysteine at the transition state may partially neutralize the cation that develops in the oxocarbenium intermediate during the ADP–ribosyltransferase reaction. This is much less the case during the NAD^+–glycohydrolyze reaction. The equivalent *N*-glycosidic bond orders in the transition states of NAD^+–glycohydrolyze and ADP–ribosylation suggest that the enzyme mechanism for the formation of the transition-state intermediate may be similar for both reactions. However, the much stronger participation of the cysteine thiol compared with water in the nucleophilic attack suggests that the enzyme exerts a somewhat different effect on the acceptor substrates.

F. The Catalytic Residues of PTX

Two catalytic amino acid residues have so far been identified in the S1 subunit: Glu-129 and His-35. It has been proposed that, in the NAD^+–glycohydrolysis reaction, a water molecule is activated by His-35 (Antoine and Locht 1994). This activation, however, does not lead to a full ionization of water

(Scheuring and Schramm 1997a). In contrast, His-35 may perhaps induce full ionization of the nucleophilic thiol of the cysteine in the G protein (Fig. 5) (Scheuring and Schramm 1997b), which may explain why alterations of His-35 have a much more pronounced effect on the ADP–ribosyltransferase activity than on the NAD^+–glycohydrolase activity of PTX (Xu et al. 1994). Whereas a change from His-35 to glutamine in a truncated recombinant S1 resulted in an approximately 50-fold reduction in the k_{cat} value of the NAD^+–glycohydrolase activity (Antoine and Locht 1994; Xu et al. 1994), the same change within the full-length S1 molecule resulted in a 500-fold reduction of the k_{cat} value of the ADP–ribosyltransferase activity (Xu et al. 1994). Neither the K_m values for NAD^+ nor the affinity for the acceptor substrate were significantly altered by this amino acid change. More drastic changes, such as a change to alanine or proline, had more dramatic effects on the enzyme activities, although the affinity for NAD^+, as measured by K_m determination or by photo-cross-linking to NAD^+, was not altered by these substitutions.

The finding that glutamine substitutions had a less dramatic effect than alanine or proline substitutions suggests that His-35 functions through hydrogen bonding, since glutamine can retain some hydrogen-bonding capacity, whereas alanine and proline do not. Glutamine can mimic some of the hydrogen bonding of the ε-*N* of histidine, whereas asparagine can mimic that of the δ-*N*, but neither of them has the proton-transfer capability of histidine. A substitution of His-35 by asparagine resulted in a further decrease in enzyme activity compared with the glutamine substitution (Antoine and Locht 1994), suggesting that the ε-*N* rather than the δ-*N* of the histidine imidazole is important for catalysis. Since there is no evidence for true proton abstraction from the water molecule in the NAD^+–glycohydrolase reaction (Scheuring and Schramm 1997a), the ε-*N* of the histidine imidazole is probably more involved in polarization of the water molecule than in its ionization.

The k_{cat} for NAD^+–glycohydrolysis varied slightly over a range of 2.5 pH units, with an estimated pK_a between 6.5 and 7 as deduced from the $\log(k_{cat})/K_m$ ratios. This slight variation in k_{cat} as a function of pH depends on His-35 protonation, because the pH dependence is abolished when His-35 is substituted by glutamine (Antoine and Locht 1994). However, since the cysteine substrate within the G protein in the ADP–ribosyltransferase reaction is ionized by PTX (Scheuring and Schramm 1997b), and since ionized cysteine is a stronger nucleophile than non-ionized water, it comes as no surprise that alterations of His-35 have a stronger effect on ADP–ribosylation than on NAD^+–glycohydrolysis (Antoine and Locht 1994; Xu et al. 1994), supporting the role of His-35 on the acceptor substrate during catalysis.

The only other catalytic residue identified in S1 is Glu-129. This residue lies within hydrogen-bonding distance of His-35 in the active-site structure of the enzyme (Stein et al. 1994a). Despite the low degree of sequence similarities among ADP–ribosylating bacterial toxins, the Cα atoms of 46 active-site residues of PTX can be superimposed on corresponding atoms of the other toxins for which the crystal structure is known. Among the superimposable

residues, Glu-129 is strictly conserved among all these toxins, in contrast to His-35, which is not conserved in diphtheria toxin and *P. aeruginosa* exotoxin A. We have previously proposed (Locht and Antoine 1995) that the strict conservation of Glu-129 may suggest that this residue acts on NAD^+, the common substrate of all ADP–ribosyltransferases (Fig. 5). As for the other toxins, Glu-129 of PTX is essential for enzymatic activity (Locht et al. 1989). A change from Glu-129 to aspartate, thus maintaining the carboxylate group of the side chain, results in a reduction of two orders of magnitude in the catalytic rate of the NAD^+–glycohydrolase reaction (Antoine et al. 1993), whereas binding to NAD^+ was not significantly affected, as evidenced by K_m measurements of NAD^+ and by fluorescent quenching studies.

Irradiation of PTX at 254 nm in the presence of NAD^+ results in the covalent binding of the nicotinamide moiety of NAD^+ to the catalytic glutamate, similar to what has been observed for some of the other toxins (Barbieri et al. 1989; Cockle et al. 1989; Cieplak et al. 1990). The structure of the photoproduct of the diphtheria-toxin–nicotinamide adduct has been determined (Carroll et al. 1985). It consists of the nicotinamide linked via its C6 to the decarboxylated γ-methylene carbon of the catalytic glutamate, implying that the γ-methylene carbon of the catalytic residue lies close to the C6 of the nicotinamide in the active site. This allows the carboxylate group of the glutamate side chain to face the 2′ OH of the ribose (Locht and Antoine 1995). The crystal structure of NAD^+ bound to diphtheria toxin confirmed that the catalytic glutamate could be in contact with the 2′-ribo-hydroxyl of the NAD^+ (Bell and Eisenberg 1996). Shortening the side chain of Glu-129 of PTX by the substitution of this residue with aspartate would move the carboxylate group of the side chain too far from the 2′ OH of the ribose to make contact. This shortening results in reduced catalysis, suggesting that the carboxylate–2′-OH interaction may be catalytically important. This interaction may conceivably result in partial deprotonation of the 2′ OH group prior to a diol-anion formation, which would promote the formation of the oxocarbenium transition-state intermediate (Fig. 5). However, from the kinetic-isotope-effect measurements, there is no evidence for 2′-OH ionization by PTX (Scheuring and Schramm 1997a). Therefore, it is more likely that the carboxylate–2′-OH contact only results in increased polarization without true ionization.

G. Substrate Binding by PTX

The addition of increasing concentrations of NAD^+ to PTX results in quenching of the intrinsic tryptophan fluorescence of the protein (Lobban et al. 1991), suggesting that a tryptophan residue may be located close to the NAD^+-binding site of S1. A truncated, recombinant version of the S1 subunit, which contains only the first 187 residues but still expresses full NAD^+–glycohydrolase activity (Locht et al. 1987), contains only a singe tryptophan residue, at position 26. A deletion of this residue or a change to threonine

abolishes enzymatic activity (Locht et al. 1989). When Trp-26 is replaced by a more conserved amino acid, such as phenylalanine or tyrosine (Cortina and Barbieri 1989; Locht et al. 1990b), residual enzyme activity can be detected, suggesting that the aromatic side-chain of Trp-26 plays an important role. Kinetic analyses of S1 polypeptides, in which Trp-26 is replaced by tyrosine or phenylalanine, indicated that these substitutions resulted in a substantially increased K_m, whereas the k_{cat} values were not significantly different from that of the original enzyme. It appears, therefore, that the protein that contains tryptophan at position 26 more efficiently binds NAD^+ than the two other analogues. In the three-dimensional structure of PTX, Trp-26 is located at some distance from the catalytic center defined by His-35 and Glu-129, but its location is consistent with a role in NAD^+ binding (Stein et al. 1994a). Given the importance of the aromatic side-chain of Trp-26, it is conceivable that, similar to other proteins, the tryptophan side-chain interacts with the NAD^+ substrate by stacking of its indole ring on one of the aromatic rings of NAD^+.

The indole ring of Trp-26 is located near the α-carbon of Cys-41. This cysteine is involved in a disulfide-bond formation with Cys-201. However, the expression of ADP–ribosyltransferase activity requires this bond to be reduced (Moss and Hewlett 1983). Replacement of Cys-41 by other amino acids reduces the enzyme activities of S1 essentially by increasing the K_m of NAD^+, consistent with the notion that this residue is close to the NAD^+-binding site (Locht et al. 1990a). However, alterations of Cys-41 have a less drastic effect than changes of Trp-26, indicating that the role of Cys-41 is less critical than that of Trp-26. Molecular modeling of the NAD^+ analogue ApUp in the active site of the enzyme suggests that the region near Cys-201 has to move to allow access of the substrates, which also may explain the requirement of reduction of S1 for the expression of enzyme activities.

The opening of the active-site pocket by the reduction of the disulfide bond allows the Giα protein to interact with the C-terminal region of the S1 subunit. Although the first 187 amino acids of S1 are sufficient for the expression of full NAD^+–glycohydrolase activity, the region downstream of residue 187 is required for full ADP–ribosyltransferase activity (Locht et al. 1990b). Better-defined studies have shown that the region between residues 180 and 219 confers high-affinity binding to the G protein (Cortina et al. 1991), and it has been suggested that the residues located between positions 195 and 219 primarily interact with the β and γsubunits of the G proteins. Deletion of the last 40 amino acid residues from S1 not only results in decreased G-protein binding (as evidenced by an increase in the K_m for the G protein transducin) but also decreases the catalytic rate of the ADP–ribosyltransferase while maintaining normal levels of NAD^+–glycohydrolase activity. This observation is consistent with the need for correct positioning of the acceptor-substrate cysteine in order to optimally catalyze the ADP–ribosyltransferase reaction.

Other important residues that have been implicated in the enzyme activities of S1 include Arg-9. Although the role of Arg-9 has not yet been defined, alterations of this residue, even by replacement with the conservative

lysine, dramatically decrease both NAD^+–glycohydrolase and ADP–ribosyltransferase activities (Burnette et al. 1988). The side chain of Arg-9 projects into the active-site cleft (Stein et al. 1994a), and the Arg-9-to-lysine change decreases the ability of the S1 protein to be photolabeled by NAD^+ (Cieplak et al. 1990), suggesting that it affects NAD^+-binding. Interestingly, Arg-9 lies in a region of S1 that is very similar to the A subunits of cholera toxin and *E. coli* heat-labile toxin (Locht and Keith 1986), two ADP–ribosylstransferases that share many properties with PTX.

H. Conclusions

PTX is the most complex bacterial toxin known. As a member of the A-B toxin family with ADP–ribosyltransferase activity, it is a major virulence factor produced by *B. pertussis* and an important component of whooping cough vaccines. Although vaccines against whooping cough have been available since the 1940s, side effects have, in the last two decades, decreased confidence in the first-generation vaccines. Studies on the structure–function relationship of PTX have greatly helped in the development of new-generation vaccines. The identification of amino acid residues involved in the enzymatic activities (Pizza et al. 1989) and, subsequently of those involved in receptor binding (Lobet et al. 1993), has led to the design of genetically detoxified PTX analogues, which have been proven to be useful components of new acellular pertussis vaccines. Such vaccines have already been tested in children and been found to be protective and to induce fewer unwanted side effects. The genetic engineering of PTX has also shown promise for the development of attenuated live *B. pertussis* vaccines capable of protecting after a single intranasal administration (Mielcarek et al. 1998).

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CHAPTER 9

Pertussis Toxin as a Pharmacological Tool

B. NÜRNBERG

A. Introduction

During the last two decades, bacterial toxins, e.g., pertussis toxin (PT), have become powerful pharmacological agents to identify and characterize biochemical entities involved in guanine-nucleotide-sensitive cellular responses elicited by hormones or neurotransmitters (BOURNE et al. 1990, 1991; WESS 1998). PT in combination with radiolabeled nicotinamide adenine diphosphate cation (NAD^+) or analogs thereof allowed probing of target proteins even in crude extracts. This sensitive technique has facilitated detection, isolation, identification and functional characterization of various members of the family of heterotrimeric regulatory guanosine triphosphate (GTP)-binding proteins, i.e., G proteins (MILLIGAN 1988; GILMAN 1995). Moreover, even in the era of genetic targeting approaches, such as antisense-oligonucleotide strategies or the gene-"knock-out" technology, PT is appreciated as a valuable tool in cell biology for discovering novel G-protein-coupled signaling pathways (GUTKIND 1998; LEFKOWITZ 1998).

PT, a 105-kDa, hexameric bacterial exoenzyme produced by *Bordetella pertussis*, was originally implicated in the pathogenesis of whooping cough (PITTMAN 1984). The molecular structure and mode of catalytic activity of PT are in accordance with the A-B model of various toxins, including cholera and diphteria toxin, which share a common enzymatic activity, i.e., the transfer of an adenosine diphosphate (ADP)–ribose moiety from NAD to different amino acids (LOCHT, this volume). PT consists of two components: the enzymatically active A protomer (consisting of a single polypeptide chain) and the pentameric B oligomer (TAMURA et al. 1982). The B component is involved in binding to the surface of eukaryotic target cells and, presumably, in translocation of the toxin across the plasma membrane. Once inside the cell, the enzymatically active A component needs to undergo activation that depends on reduced gluthathione; this effect can be mimicked in a cell-free system with sulfhydryl-reducing agents. Therefore, use of holoenzyme is mandatory for treatment whole cells, whereas the preactivated A protomer is able to modify cellular proteins when injected or infused into cells or in cell homogenates or purified protein preparations. Enzymatic activity of PT results in pathological responses of the infected cells, resulting in increased susceptibility of the organism to various signaling molecules, including histamine and serotonin. A

hallmark of PT action following injection is hyperinsulinemia despite marked hypoglycemia, which is due to sustained potentiation of insulin secretion from pancreatic islets and refractoriness to adrenaline. Accordingly, PT was given the synonym islet-activating protein. PT also alters the immune system, causing lymphocytosis and leucocytosis. All these actions have been explained by the profound and irreversible interference of PT with G proteins.

The use of PT as an experimental tool has greatly influenced our current understanding of transmembrane signaling. In fact, seven transmembrane ligand-binding proteins, i.e., G-protein-coupled receptors (GPCRs), in concert with their transducers, effectors and regulators, have emerged as the dominant signal-transduction machinery in mammalian cells (WATSON and ARKINSTALL 1994; HELDIN and PURTON 1996). This concept is underscored by the fact that approximately 90% of all hormones and neurotransmitters and two out of three currently prescribed, established drugs are assumed to exert their actions by interfering with the superfamily of heptahelical GPCRs (GUDERMANN et al. 1995). Accordingly, hundreds of receptor ligands exhibiting a remarkable chemical heterogeneity use this system through interaction with more than 1000 receptors (HELMREICH and HOFMANN 1996; JI et al. 1998; WESS 1998). In turn, ligand-occupied GPCRs couple to dozens of signal-transducing G-protein isoforms, which are membrane-associated heterotrimers attached to the cytoplasmatic surface. They belong to the superfamily of GTP-binding proteins or GTPases and are extremely conserved proteins throughout the animal kingdom, with considerable homology to plant counterparts (WILKIE et al 1992). In contrast to enzymatically active receptors or ligand-operated ion channels, the GPCRs lack effector domains. Hence, it is the receptor-activated G protein which switches the incoming signal into intracellular second messengers by modulating other enzymes or ion channels (GETHER and KOBILKA 1998; HAMM 1998).

Recently with the aid of PT as an experimental tool, evidence emerged that has extended our thinking about G proteins beyond their classical role as transducers of extracellular signals. G proteins, which have been found in cytosolic compartments (including endomembranes and vesicles), have now been recognized to regulate membrane-trafficking and vesicular-transport mechanisms of the cell (HELMS 1995; NÜRNBERG and AHNERT-HILGER 1996). Therefore, G proteins are considered to be universal switches involved in modulation of housekeeping and intra- and intercellular communicative functions of the cell.

PT-mediated ADP–ribosylation of G proteins represents a virtually irreversible modification that appears to be stable for the lifetime of the protein both in vivo and in vitro. This short overview pursues the concept of PT as an appropriate pharmacological tool for structural, functional and cell-biological studies. It represents a quick and cheap G-protein-"knock-out" approach. Consequently, here I will focus on three aspects of PT function: (1) as a probe to understand structure–function relationships of G proteins, (2) as an appropriate tool to discover G-protein-dependent signaling pathways, and (3) as an

agent assigning G-protein actions to particular G proteins. Those who are interested in more complete reviews or other aspects of PT are referred to numerous excellent previous overviews (UI 1984; MOSS and VAUGHAN 1988; GIERSCHIK and JAKOBS 1993; YAMANE and FUNG 1993; LOCHT 1997).

B. Molecular Aspects of PT Activity on G Proteins

I. General Considerations

PT action is not limited to whole cells but also exerts its effects in fractionated cell preparations or purified protein solutes devoid of lipid membranes. It covalently modifies α subunits of a subset of heterotrimeric G proteins, which are traditionally classified as PT-sensitive G proteins. This modification appears to be irreversible though, under certain experimental conditions, mercury ions specifically remove the transferred moiety from $G\alpha$ (MEYER et al. 1988; NÜRNBERG et al. 1994). PT-sensitive G proteins are almost congruent with the G_i subfamily members of heterotrimeric G proteins, including three forms of G_i, two splice variants of G_o, two transducins and the gustatory G protein, G_{gust} (but not G_z and the longer splice variant of G_{i2}; NÜRNBERG 1997). Additionally, detected PT substrates, such as a fourth, 43-kDa $G\alpha_i$ isoform (IYENGAR et al. 1987) or a third $G\alpha_o$ isoform (NÜRNBERG et al. 1994; WILCOX et al. 1995), which presumably represent a splice variant of the $G\alpha_{i3}$ transcript (CARTY and IYENGAR 1990) and a deamidated $G\alpha_{o1}$ (i.e.,$G\alpha_{o3}$; MCINTIRE et al. 1998; EXNER et al. 1999), respectively. The primary structures of some other PT substrates still remain to be identified. These unknown entities (1) copurify with known PT-sensitive $G\alpha$ isoforms, e.g., $G\alpha_{i1}$ or $G\alpha_{i2}$, (2) are sensitive to antisera directed against PT-sensitive $G\alpha$ isoforms, and (3) exhibit apparent molecular masses ranging between 39 kDa and 45 kDa (NÜRNBERG et al. 1994). Therefore, it is possible to speculate that they are unidentified splice variants (or other modified versions) of PT-sensitive $G\alpha_i$-subfamily members. In contrast, most PT-insensitive $G\alpha$ isoforms belong to three other G-protein subfamilies: (1) those harboring G_s proteins, which are sensitive to cholera toxin (CT) and best known for their ability to enhance formation of cyclic adenosine monophosphate by stimulating adenylyl cyclases (HOL, this volume; Moss, this volume); (2) those of G_q proteins, which are activators of phospholipase C-β-isoforms; and (3) those of the poorly characterized $G_{12/13}$ proteins (NÜRNBERG et al. 1995). Nonetheless, it should be kept in mind that the classification of G proteins into four subfamilies is solely based on amino acid homology of $G\alpha$ subunits, which bind guanine nucleotides and exhibit GTPase activity, while the heterogeneity of the enzymatically inactive $G\beta$ and $G\gamma$ subunits do not contribute to this nomenclature. However, as outlined below, PT intervention of a signaling pathway does not allow one to distinguish between the $G\alpha$ subunit and the $G\beta\gamma$ complex of the PT-modified G protein responsible for a cellular effect.

II. PT-Sensitive G Proteins

1. Mechanism of PT Action

PT catalyzes both hydrolysis of NAD^+ and the transfer of the resulting ADP–ribose moiety to those $G\alpha$ subunits that have a cysteine residue located four positions upstream from the C-terminus (see above and Table 1; WEST et al. 1985). Introduction of a cysteine at this position in a PT-insensitive $G\alpha$ like $G\alpha_s$ turns it into a PT-sensitive isoform, though it was found to be a poor substrate for the toxin (FREISSMUTH and GILMAN 1989). The C-terminal amino acid motif of $G\alpha$ required for PT-mediated ADP–ribosylation is similar to a consensus site for a post-translational sequence of reactions comprising proteolysis, carboxymethylation and isoprenylation of $G\alpha$ subunits or various monomeric GTPases. Notably, the C-terminal aromatic amino acid of the PT-recognition motif determines the sensitivity of $G\alpha$ towards PT and the resistance against endogenous lipidation (NEER et al. 1988; JONES and SPIEGEL 1990). In addition, some adjacent residues next to the target cysteine appear to be important for PT sensitivity of $G\alpha$ (AVIGAN et al. 1992). Synthetic peptides resembling C-termini of $G\alpha_i$ isoforms not only inhibit PT-mediated ADP–ribosylation of G proteins in a competitive, concentration-dependent manner but also serve as substrates for PT (GRAF et al. 1992; SCHEURING and SCHRAMM 1997). As outlined below, this finding was somewhat surprising, since it was suggested that $G\alpha$ is only marginally modified by PT in the absence of

Table 1. C-terminal amino acid sequences of $G\alpha$ isoforms belonging to four subfamilies, i.e., $G\alpha_i$, $G\alpha_s$, $G\alpha_q$ and $G\alpha_{12}$

Isoform	Amino acid sequence		
α_{i1}	II	KNNLK	DCGLF
α_{i2}	II	KNNLK	DCGLF
α_{i2l}	AP	APPLS	SDSVP
α_{i3}	II	KNNLK	ECGLY
α_{o1}	II	ANNLR	GCGLY
α_{o2}	II	AKNLR	GCGLY
α_{o3}	II	ADNLR	GCGLY
α_{tr}	II	KENLK	DCGLF
α_{tc}	II	KENLK	DCGLF
α_{gust}	II	KENLK	DCGLF
α_z	II	QNNLK	YIGLC
α_q	IL	QLNLK	EYNLV
α_{11}	IL	QLNLK	EYNLV
α_{14}	IL	QLNLR	EFNLV
$\alpha_{15/16}$	VL	ARYLD	EINLL
α_{ss}	IQ	RMHLR	QYELL
α_{ls}	IQ	RMHLR	QYELL
α_{xls}	IQ	RMHLR	QYELL
α_{olf}	IQ	RMHLK	QYELL
α_{12}	IL	QENLK	DIMLQ
α_{13}	IL	HDNLK	QLMLQ

$G\beta\gamma$ complexes (Watkins et al. 1985). In addition to $G\beta\gamma$, other cofactors supporting PT activity under in vitro conditions include adenine nucleotides, phospholipids and detergents (Moss et al 1986). The exact role of $G\beta\gamma$ complexes in enhancing the velocity of the PT-catalyzed enzymatic reaction is still unclear, but it is unlikely that they simply prevent $G\alpha$ from denaturation (Gierschik and Jakobs 1993). It is assumed that $G\beta\gamma$ facilitates PT-mediated ADP–ribosylation in a catalytic manner by cycling among different molecules of $G\alpha$ (Casey et al. 1989; Ueda et al. 1994). Since substoichiometric amounts of $G\beta\gamma$ are supportive, rates of PT-catalyzed ADP–ribosylation do not permit direct assessment of the relative affinities of $G\beta\gamma$ for $G\alpha$. It has also been reported that, under certain experimental conditions, the proportion of $G\alpha$ that is ADP–ribosylated is proportional to the amount of $G\beta\gamma$ added (Graf et al. 1992). In fact, PT-mediated ^{32}P-ADP–ribosylation of a known amount of purified $G\alpha_i$ in the presence of unknown concentrations of $G\beta\gamma$ can be used to quantify the amount of functionally active $G\beta\gamma$ (Iniguez-Lluhi et al. 1992).

2. G-Protein Specificity

G proteins are heterotrimers composed of three different subunits, termed α, β and γ, with molecular masses of approximately 39–45, 35–39 and 6–8 kDa, respectively (Fig. 1; Nürnberg et al. 1995). For PT-mediated ADP–ribosylation of $G\alpha$, the functional interaction between $G\alpha$ and the tightly associated $G\beta\gamma$ complex appears to be crucial (Gierschik and Jakobs 1993). This feature is exploited to use PT-mediated ADP–ribosylation as a standard assay to assess the interactions of these two functional G-protein subunits. In principle, parameters affecting the affinities between G-protein subunits should also influence the ability of PT to modify $G\alpha$. For example, ADP–ribosylation of bovine brain $G\alpha_{o1}$ requires higher $G\beta\gamma$ concentrations than ADP–ribosylation of $G\alpha_{o2}$ or $G\alpha_{i2}$ purified from the same source (Huff and Neer, 1986; Nürnberg et al 1994). This observation coincides with the fact that purified $G\alpha_{i2}$ and $G\alpha_{o2}$ have a lower affinity for bovine brain $G\beta\gamma$ complexes than $G\alpha_{o1}$ does (Sternweis and Pang 1990; Exner et al. 1999). Obviously, changes in the primary structure of the $G\alpha$ N-terminus, as the site of interaction with $G\beta\gamma$, affect PT sensitivity (Wedegaertner et al. 1995). This includes absence of a covalently linked myristate as well as removal of approximately 20 N-terminal amino acids of $G\alpha$, which leads to diminished ADP–ribosylation (Navon and Fung 1987; Neer et al. 1988; Jones et al. 1990; Linder et al. 1991). In contrast, an additional covalently linked (albeit labile) palmitate seems to have no significant impact on PT-mediated ADP–ribosylation.

More obvious, the structure of the C-terminal region harboring the target cysteine affects the sensitivity to PT (see above). Therefore, it was not surprising that a novel $G\alpha_o$ isoform, $G\alpha_{o3}$, which only differs from $G\alpha_{o1}$ by exchange of a single amino acid (^{346}Asn to Asp), was suggested to be a worse substrate for PT than $G\alpha_{o1}$ (Nürnberg et al. 1994). Studies on the initial rates of PT-mediated ADP–ribosylation of purified $G\alpha_{o1}$ and $G\alpha_{o3}$ revealed

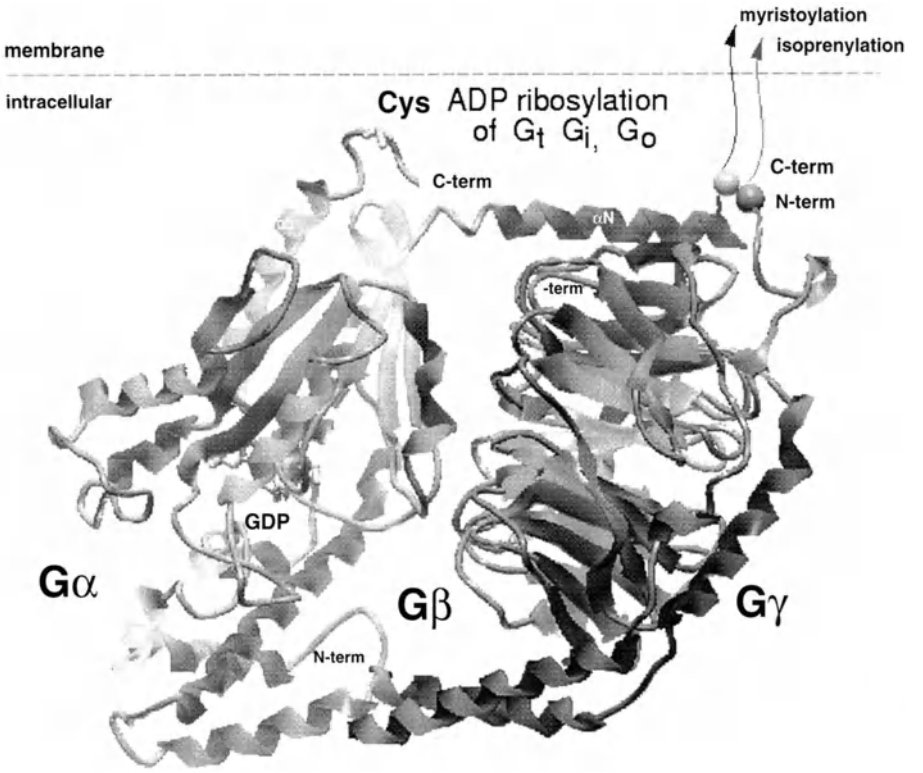

Fig. 1. Structure of a pertussis-toxin (PT)-sensitive heterotrimeric G protein based on $G\alpha_i\beta\gamma$. $G\alpha_i$ in its inactive, guanosine diphosphate-bound conformation, is complexed with $G\beta$ and $G\gamma$. The PT-sensitive C-terminal cysteine of $G\alpha$ is indicated. Orientations of the proteins towards the membrane is speculative but supported by the close proximity of myristoylation and isoprenylation sites of $G\alpha$ and $G\gamma$, respectively (data from LAMBRIGHT et al. 1995)

significant differences in the velocity of ADP–ribosylation between the two species, though no difference in maximal incorporation of ^{32}P-ADP–ribose into either protein was observed after prolonged incubation of $G\alpha$ in the presence of equimolar amounts of $G\beta\gamma$ complexes (EXNER et al. 1999). Further analysis with increasing amounts of bovine-brain $G\beta\gamma$ complexes showed that excess amounts of $G\beta\gamma$ resulted in similar maximum rates of ^{32}P-ADP–ribosylation in $G\alpha_{o1}$ and $G\alpha_{o3}$, with an approximately fivefold difference in $G\beta\gamma$ EC_{50} values. This strongly suggests that differences in PT-mediated ^{32}P-ADP–ribosylation were caused by different interactions of $G\alpha_{o1}$ and $G\alpha_{o3}$ with $G\beta\gamma$ dimers. This finding emphasizes the assumption that differences in PT-mediated ADP–ribosylation are secondary to $\alpha\beta\gamma$-heterotrimer formation rather than representing an immediate consequence of the different C-termini of $G\alpha$. In accordance with this suggestion, $G\alpha_{o1}$ and $G\alpha_{o3}$ were found to be associated with distinct $G\beta$ and $G\gamma$ isoforms, pointing to the involvement of

the C-terminus of Gα in subunit association (Wilcox et al. 1995; Exner et al. 1999). Further support comes from cross-linking studies employing derivatives of NAD^+, e.g., ^{32}P-2-azido-NAD^+, that were used as substrates by PT (Vaillancourt et al. 1990, 1995). These studies revealed intramolecular interactions of Gα N- and C-termini and intermolecular cross-linking of the C-terminus of Gα with Gγ. Other derivatives used for structural analysis of Gα comprised the fluorescently labeling nicotinamide-1-N^6-ethenoadenine dinucleotide, which was accepted as a substrate by PT, and *N*-(3-iodo-4-azidiphenylpropionamide-*S*-(2-thiopyridyl)cysteine, which bound to the C-terminal PT-sensitive cysteine of Gα. In contrast, *N*-ethylmaleimide and other sulfhydryl-alkylating reagents, which also prevented ADP–ribosylation by PT, appeared to bind to a cysteine in the N-terminal third of Gα, thereby most likely inhibiting interaction of alkylated Gα with Gβγ (Dhanasekaran et al., 1988; Hingorani and Ho 1988; Kaslow et al. 1989; Hoshino et al. 1990). The latter finding points again to the hetrotrimeric G protein as the preferred substrate for PT, emphasizing the importance of functional interaction of Gα with the Gβγ complex for PT-mediated modification of Gα.

III. PT as a Tool with which to Study G-Protein-Subunit Composition

Accordingly, integrity of the Gβγ complex is indispensable for proper function of PT. For instance, PT has been helpful in screening for Gβγ combinations unable to dimerize, such as $G\beta_2\gamma_1$, which consequently fails to support ADP–ribosylation of Gα (Iniguez-Lluhi et al. 1992). This strategy is facilitated by the fact that all PT-sensitive Gα isoforms complex with most Gβγ dimers studied so far. Nevertheless, depending on the Gα isoform used as the substrate for PT action, differences in efficacy and potency of various native and recombinant Gβγ isoforms supporting PT-catalyzed ADP–ribosylation have been found. While transducin Gα was equally well modified by PT in the presence of transducin βγ from rod outer segments and placentar Gβγ, recombinant $G\alpha_{i3}$ was a better substrate for PT when co-incubated with placentar Gβγ than with transducin βγ (Fawzi et al. 1991; Graf et al. 1992). In the latter study, Gβγ complexes from red blood cells or bovine brain had intermediate effects (Graf et al. 1992). In addition, purified recombinant Gβγ dimers of defined composition were tested (Iniguez-Lluhi et al 1992; Müller et al. 1993; Ueda et al. 1994). These studies revealed that differences in the abilities of the purified proteins to support ADP–ribosylation of $G\alpha_o$ were negligible. In contrast, recombinant $G\beta_1\gamma_1$ significantly differed from all other Gβγ dimers tested when recombinant $G\alpha_{i1}$ was employed. Furthermore, various recombinantly generated Gβγ dimers lacking the isoprenoid modification of Gγ failed to support PT-mediated ADP–ribosylation of Gα, corresponding to a significantly decreased ability of non-prenylated Gβγ to complex with Gα (Wedegaertner et al. 1995). Similarly, carboxymethylation of the C-terminal cysteine of Gγ has been predicted to stabilize the association of guanosine

diphosphate (GDP)-bound Gα and G$\beta\gamma$. However, its relevance for PT-mediated ADP–ribosylation is still a matter of research. While several studies have described an enhanced ADP–ribosylation of Gα_i and Gα_t in the presence of various methylated G$\beta\gamma$ dimers compared with non-methylated counterparts, one report found no difference on PT-catalyzed modification of Gα_{i1} after demethylation of G$\beta_1\gamma_2$ (FUKADA et al. 1994; PARISH and RANDO 1994; PARISH et al. 1995; ROSENBERG et al. 1998).

In conclusion, these data strongly suggest that structures of all three G-protein subunits, i.e., Gα, Gβ and Gγ, and differences in their post-translational processing affect the ability of PT to modify Gα. Putative reasons certainly include differences in the affinities of Gαs for G$\beta\gamma$s and other parameters (see above). Therefore, PT may serve as a useful tool for study of the functional consequences of novel post-translational modifications on G-protein subunits, including heterogeneous processing and phosphorylation of Gγ subunits (FUKADA et al. 1990; COOK et al. 1998; YASUDA et al. 1998; UEDA et al. 1999).

In this context, a fifth Gβ isoform, Gβ_5, has recently attracted much attention. It exhibits a significant difference in its amino acid sequence, with only 53% identity to other Gβ subunits, whereas the deduced amino acid sequences of Gβ_{1-4} display a high degree of homology, with at least 79% identity (WATSON et al. 1994). Consistent with the idea that the low level of homology may correspond to distinct functions of Gβ_5, a differential coupling pattern of Gβ_5 to receptors and effectors as compared with other Gβ isoforms was found (ZHANG et al. 1996; BAYEWITCH et al. 1998a, 1998b; LINDORFER et al. 1998). Furthermore, binding experiments with immobilized G$\beta\gamma$ dimers suggested a selective binding of G$\beta_5\gamma_2$ to G$\alpha_{q/11}$ proteins but not to PT-sensitive G$\alpha_{i/o}$ or Gα_s isoforms (FLETCHER et al. 1998). In agreement with these reports, Gβ_5-immunoreactive proteins predominantly co-eluted from an anion exchange column with G$\alpha_{q/11}$ rather than G$\alpha_{i/o}$ proteins subsequent to cholate extraction from bovine-brain membranes (EXNER and NÜRNBERG, unpublished). Notably, immunocytochemical studies using specific antisera in rat brain showed a differential distribution of Gβ_5 and G$\alpha_{q/11}$, arguing against an immediate physiological relevance of these findings (AHNERT-HILGER and NÜRNBERG, unpublished). Consequently, as an unequivocal proof of functional subunit interaction, G$\beta_5\gamma_2$ was studied for its ability to support ADP–ribosylation of Gα_i. In fact, membranes from baculovirus-infected Sf9 cells containing recombinant Gα_{i1} and G$\beta_5\gamma_2$ or G$\beta_1\gamma_2$ showed a marked increase of Gα_{i1}–ADP–ribosylation compared with membranes lacking either G$\beta\gamma$ dimer (MAIER et al., 2000). Moreover, functionally active recombinant G$\alpha_{i1\text{-}His}\beta_5\gamma_2$ have been isolated from Sf9 cell membranes by affinity chromatography, and G$\beta_5\gamma_2$ dimers were specifically released from immobilized G$\alpha_{i1\text{-}His}$ following incubation with AlF_4^-, an activator of heterotrimeric G proteins. These findings emphasize the aforementioned interaction of PT-sensitive Gα subunits with G$\beta\gamma$ complexes of different composition. Surprisingly, in contrast to other G$\beta\gamma$ dimers, purified preparations containing Gβ_5 lost their ability to support

PT-mediated ADP–ribosylation. Subsequent search for possible causes revealed that $G\beta_5$ exhibits a unique tendency to dissociate from $G\gamma$ (Fletcher et al. 1998; Babich et al., in preparation). As a $G\gamma$-free $G\beta_5$ monomer, it is soluble in the absence of detergents and unable to interact with $G\alpha_{i/o}$ proteins (Babich et al., in preparation). The physiological relevance of this remarkable decreased affinity of $G\beta_5$ for $G\gamma$ is currently unclear, though a recent report has shown the association of $G\beta_5$ with G-protein γ-subunit-like domains, which was found in various regulator of G-protein-signaling (RGS) proteins, e.g., RGS7 and RGS11 (Snow et al. 1998; Levay et al. 1999).

C. Functional Consequences of PT Activity

I. PT-Affecting Receptor–G-Protein–Effector Coupling

The PT-catalyzed covalent modification of $G\alpha$ has immediate consequences for the functional interaction of G proteins with receptors. To fulfill their roles as cellular switches, G proteins permanently cycle between activated and inactivated states (Fig. 2; Gilman 1987, Sprang 1997; Hamm 1998). Transition from the inactive to the active state is catalyzed by GPCRs and coincides with the release of GDP from $G\alpha$ and the uptake of GTP. Mg^{2+} is very tightly associated with GTP and $G\alpha$ in this complex and is required for activation of the $G\alpha$ subunit and for subsequent hydrolytic activity (Higashijima et al. 1987). Upon G-protein activation, the $G\alpha$ subunit changes its conformation, which decreases its affinity for $G\beta\gamma$, allowing dissociation of the heterotrimer (Runnels and Scarlate 1999). Both the activated $G\alpha$ subunit and the $G\beta\gamma$ complex modulate effector proteins until hydrolysis of the $G\alpha$-bound GTP terminates signaling. The GTPase activity of the $G\alpha$ subunit is regulated by some effectors and other specialized components called regulators of G-protein signaling, i.e., RGS proteins (Berman and Gilman 1998; Wieland and Chen 1999). Following GTP hydrolysis, the inactive GDP-bound $G\alpha$ subunit dissociates from the effector and reassociates with the $G\beta\gamma$ complex to form a $G\alpha\beta\gamma$ heterotrimer. Therefore, it should be kept in mind that the active state of $G\alpha$ governs the signaling kinetics of the $G\beta\gamma$ complex; in other words, $G\alpha$ can be considered as a negative regulator of $G\beta\gamma$ function (Hamm 1998).

Ligand-bound GPCRs stimulate activation of the G protein through interaction with distinct cytoplasmatic segments, catalyzing the release of bound GDP followed by high-affinity binding of cytosolic GTP (Ji et al. 1998). This interaction is blocked by PT. Since the modified cysteine is located four residues upstream from the C-terminus, PT-catalyzed ADP–ribosylation prevents interaction of the transmembrane receptor with the G protein, which is anchored to the cytoplasmic surface of the membrane. This results in functional uncoupling of the receptor and the G protein (Gierschik and Jakobs 1993). By doing so, the receptor becomes unable to regulate effector pathways. These include an increasing number of second-messenger-generating enzymes,

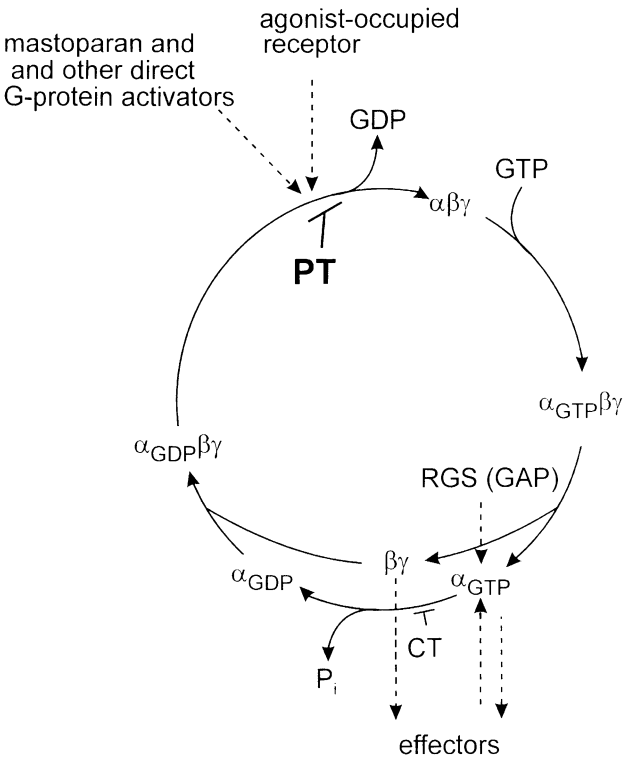

Fig. 2. Activation/inactivation cycle of G proteins. α, β And γ represent the Gα, Gβ and Gγ subunits of the G protein. Pertussis toxin (*PT*) functionally uncouples the G protein from the receptor, whereas cholera toxin (*CT*) abolishes the guanosine triphosphatase (GTPase) activity of the G protein. Regulators of G protein signaling (*RGS*) and GTPase-activating proteins (*GAPs*) modulate the intrinsic GTPase activity of Gα

transporters and ion channels, including various adenylyl cyclases, phospholipases A and C-β, phosphoinositide-3 kinases β and γ, cyclic guanosine monophosphate phosphodiesterases and channels for calcium, potassium and sodium. Vice versa, G proteins activated by GPCRs show a decreased sensitivity to PT. Therefore, quantification of ^{32}P-ADP-labeling of G proteins following incubation in the presence of PT and receptor agonists has been used as a read-out system with which to examine coupling of receptors to individual G proteins (OKAMOTO et al. 1991; KÖRNER et al. 1995). Agonist-bound GPCRs activate G proteins, thereby inducing dissociation of subunits, which leads to an impaired capability of PT to ADP-ribosylate the coupling Gα. Hence, PT is considered to be an important pharmacological tool with which to examine $G_{i/o}$-sensitive receptor–effector signal-transduction pathways (see below). Though it is well established that PT modification does not alter enzymatic properties of the G protein, basal GTPase activity and GTPγS binding

is reduced in membranes of PT-treated cells (Gierschik and Jakobs 1993). These observations probably reflect the ability of an "empty" (i.e., agonist-free) receptor to exert a tonic stimulation of G-protein activity, a phenomen also prevented by treatment of receptors with inverse agonists (Costa and Herz 1989).

PT-modified G proteins are still able to exchange GDP for GTP and to dissociate into activated $G\alpha$ and $G\beta\gamma$. Therefore, both regulatory subunits of the GTPγS-activated PT-modified G protein keep their ability to interact with effectors. Nevertheless, the time required to observe the maximal effect by GTPγS can be markedly increased, probably due to steric hinderance of the ADP–ribose moiety at the C-terminus of $G\alpha$ (Jakobs et al. 1984; Ahnert-Hilger et al. 1998). Nonetheless, it is currently unclear whether these slowed kinetics are also inherent in effector modulation by $G\beta\gamma$ dimers released from a GTP-activated, PT-modified G protein.

II. PT-Affecting Receptor-Independent Activation of G Proteins

Peptides bearing cationic regions (like mastoparan, a tetradecapeptide isolated from the venom from wasps or hornets) function as GPCR surrogates that initiate $G_{i/o}$ signaling (Nürnberg et al. 1999). With striking similarity to receptor-induced G-protein activation, mastoparan interacts with N- and C-terminal regions of $G\alpha$, thereby activating G proteins by stimulating the release of GDP from $G\alpha$ without altering the rate of hydrolysis of bound GTP (Higashijima et al. 1988, 1991; Weingarten et al. 1990). In addition, it has been shown that mastoparan blocks the ability of G_o to increase the affinity of muscarinic agonists, suggesting that mastoparan and the receptor may compete for a common binding site on G_o (Higashijima et al. 1990). In accordance with the assumption of a close mechanistic parallel between the activation of G proteins by mastoparans and GPCR, mastoparan-induced G-protein activation is also affected by PT (Fig. 3). The stimulatory effect of mastoparan cannot completely be blocked by PT, though mastoparan predominantly activates $G_{i/o}$ proteins and is less effective in stimulating G_t, G_s or G_q proteins (Sukumar et al. 1997). The partial PT insensitivity of mastoparan is most likely due to stimulation of nucleoside diphosphate kinases and monomeric GTPases in membranes (Koch et al. 1991, 1992; Klinker et al. 1994). Notably, both cationic amphiphilic α-helical peptides and various established drugs and experimental compounds directly activate PT-sensitive G proteins in a receptor-independent fashion (Nürnberg et al. 1999). In contrast to receptors, these compounds do not require the presence of $G\beta\gamma$ complexes to exert their stimulatory activity. These systematically designed compounds exhibit a much more selective action, corresponding to the ability of PT to completely block their stimulatory action on G proteins (Fig. 3; Leschke et al. 1997; Breitweg-Lehmann et al., unpublished). Therefore, PT has become an important tool with which to screen new chemical entities for a selective mode of action on G proteins (Nürnberg et al. 1999).

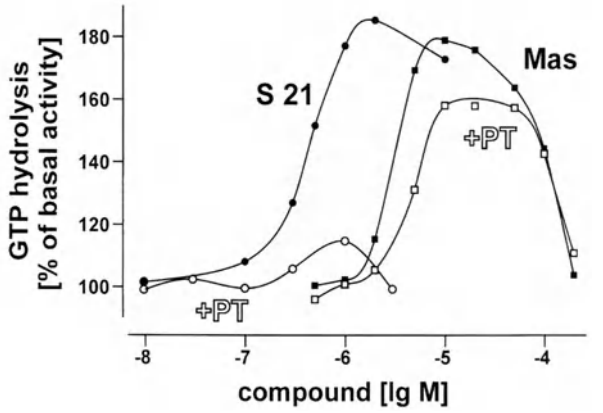

Fig. 3. Activation of HL-60 membrane proteins by mastoparan (Mas) and compound 21 (S21). Membranes of HL-60 cells incubated in the absence (*filled symbols*) or presence (*open symbols*) of pertussis toxin (*PT*) were treated with increasing concentrations of Mas or S21 [*N*-(2,5-diaminopentyl)dodecylamine]. Guanosine triphosphate (GTP) hydrolysis was quantified by measuring the amount of ^{32}P phosphate released from [γ^{32}P]GTP

III. Use of PT in Studying Cellular Signal Transduction

PT ADP–ribosylates target G proteins in most cultured mammalian cells reliably, i.e., almost quantitatively (NÜRNBERG 1997). Nevertheless, it has to be kept in mind that cells may respond differently to the toxin. In particular, platelets are able to resist the entry of PT, either because they lack surface binding sites for the toxin-B oligomer or because they are unable to internalize the bound toxin (BRASS et al. 1990; KOESLING and NÜRNBERG 1997). PT-sensitive G proteins expressed in insect Sf9 cells (ovary cells from the insect *Spodoptera frugiperda*; Sf9 cells are employed as an overexpression system for G proteins and other signal-transduction components by infection with recombinant baculoviruses) are also not modified by the toxin (MULHERON et al. 1994). In addition, at present, it is not clear whether sperms from various mammalian species are sensitive to PT. Therefore, it may be necessary to introduce the activated PT-A protomer directly into cells assumed to be resistant to PT. Anyway, proper function of PT in a cellular system has to be checked before drawing conclusions about PT's action on a cellular signaling system.

Despite these experimental considerations, PT represents an excellent tool with which to study the pattern of coupling of cell-surface receptors to intracellular signal transduction systems. Whereas PT was first appreciated as a tool with which to evaluate the coupling of heptahelical (and presumably some non-heptahelical) receptors to G proteins, it was subsequently found to reveal coupling of GPCRs to PT-sensitive and -insensitive G proteins (NISHIMOTO 1991; OFFERMANNS and SCHULTZ 1994).

Interestingly, while the number of effector functions of $G\beta\gamma$ complexes have exceeded those elicited by PT-sensitive $G\alpha$ subunits, almost every cellular effect sensitive to PT has been speculated to be induced by $G\beta\gamma$complexes (Birnbaumer 1992; Clapham and Neer 1997). Nonetheless, it has to be kept in mind that a $G\beta\gamma$-dimer-modulating cellular effector may be released from PT-sensitive or -insensitive G proteins. For example, whereas $G\beta\gamma$ complexes released from PT-sensitive G_o proteins have been found to inhibit voltage-operated calcium channels in a membrane-delimited manner, $G\beta\gamma$ dimers from PT-insensitive G proteins were recently reported to induce stimulation of voltage-operated calcium channels via activation of phosphoiniositide-3 kinases (Herlitze et al. 1996; Ikeda 1996; Viard et al 1999).

By using PT as an efficient tool in cell biology, ample evidence strongly suggests that different receptor classes communicate with each other via downstream signaling elements, a phenomenon now commonly known as "cross-talk" (Gutkind 1998). Finally, PT is still one of the most important tools with which to study the effects of intracellular and endomembrane G proteins (Helms 1995; Nürnberg and Ahnert-Hilger 1996; Lang 1999).

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Appendix: Experimental Protocols for Using PT

I. Source of PT and Preparation of Solutions

PT (List Biological Laboratories, Campbell, Calif., USA; container with 50 μg PT, 25 μmol NaCl, 5 μmol phosphate buffer, pH 7.0) is reconstituted in a solution containing 2 M urea and 100 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.0 (final concentration of PT: 50–1000 μg/ml; stored at 4°C).

II. Treatment of Mammalian Cell Cultures with PT

For ADP–ribosylation of G proteins in cultured mammalian cells, the medium is usually supplemented with PT (usually 25–200 ng/ml) overnight.

III. Activation of PT for in Vitro ADP–Ribosylation

For activation of holoenzyme, PT (5 μl, 1 mg/ml) is co-incubated with 5 μl 10 mM adenosine triphosphate (ATP), 25 μl 40 mM dithiothreitol (DTT), and 15 μl water (37°C, 30 min). Thereafter, the mixture is diluted with 450 μl of a buffer made of 125 μl 1% bovine serum albumin (BSA) in water

(weight/volume), 50 µl 0.5 M DTT, 31 µl 40 mM ethylene diamine tetraacetic acid (EDTA), 31 µl 1 M tris(hydroxymethyl)aminomethane chloride (Tris-Cl), pH 7.5, and 1013 µl water.

IV. ADP–Ribosylation of Cell-Membrane Proteins by PT

Cell membranes (stored in aliquots of 250 µg/tube and 500 µg/tube at –70°C) are thawed on ice and washed using a microcentrifuge (13000 × *g*, 4°C, 10 min). The membrane pellet is resuspended in a buffer containing 25 mM Tris-Cl, pH 7.5, and 1 mM EDTA. The volume of the membrane suspension is adjusted to 20 µl (10–100 µg of protein) per sample. The reaction buffer consists of 1.5 µl 40 mM of EDTA, 3 µl 100 mM ATP, 0.6 µl 100 mM GTP, 1.8 µl 10% polyoxyethylene-10 lauryl ether, 6 µl 100 mM thymidine, 0.6 µl DNAse (2 mg/ml), 5 µl 1 M Tris-Cl, pH 7.5, and 0.3 µl 200 µM unlabelled NAD^+. ^{32}P-NAD^+ is added to give 500,000–1,000,000 cpm/tube (~0.25–0.5 µCi). The buffer is diluted with water to give a final volume of 30 µl/tube. ADP–ribosylations of the samples are conducted in glass tubes (~5 ml) in case the reaction is followed by precipitations with organic solvents. Each tube contains 10 µl pre-activated toxin (100 ng PT/tube) and 20 µl membrane suspension. The reaction is started by addition of 30 µl of the ^{32}P-NAD^+-containing buffer, resulting in a total volume of 60 µl/tube. ADP–ribosylation of the sample is usually conducted for 30 min at 30°C or for 20 min at 37°C while the tubes are continuously shaken.

V. ADP–Ribosylation of Isolated Proteins by PT

For ADP–ribosylation of extracted proteins, the reaction buffer is slightly different from the one used for membrane treatment. Volumes are given per assay tube: 0.75 µl 40 mM EDTA, 1.5 µl 100 mM ATP, 0.3 µl 100 mM GTP, 0.9 µl 10% polyoxyethylene-10 lauryl ether, 0.75 µl 1 M Tris-Cl (pH 7.5) and 0.15 µl 200 µM unlabelled NAD^+. ^{32}P–NAD^+ is added to reach an activity of 1000000 cpm/tube. The buffer is diluted with water to give a final volume of 10 µl/tube. Ten microliters of G-protein-containing sample is mixed with 10 µl pre-activated toxin and started by addition of another 10 µl reaction buffer (total volume 30 µl). The assay is conducted as outlined under IV.

VI. Preparation of Samples for Sodium Dodecyl Sulfate Polyacrylamide-Gel Electrophoresis

Samples subjected to analysis of labeled proteins by sodium dodecyl sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) should be devoid of detergents, and non-incorporated radioactivity should be extracted before loading gels. Therefore, the reaction is terminated by supplementing the samples with 10 µl 1.5 M NaCl (4°C) and 600 µl ice-cold acetone followed by centrifugation at 3000 × *g* (30 min, 4°C). Following sedimentation, supernatants are carefully

and completely removed by aspiration. Pellets are carefully dissolved by 1 ml of ice-cold trichloroacetic acid [TCA, 20% (volume/volume)] during gentle shaking of the tube. Exposure of the sample to TCA should be short. Therefore, centrifugation (20–25 min, 3000 × *g*, 4°C) and aspiration of the supernatant is completed in a limited time in order to avoid degradation of proteins. Afterwards, TCA and lipids are removed by adding 1 ml of ice-cold chloroform/methanol (1/1 volume/volume) to the pellet. Alternatively, diethyl ether may be used equally well but has the disadvantage that it is explosive. After centrifugation and careful aspiration as before, the samples are completely dried. Pellets are dissolved in sample buffer (25–75 µl, room temperature), thoroughly vortexed and subjected to SDS-PAGE. Samples should not be boiled before loading onto gels, because G proteins sometimes tend to aggregate under these conditions and will not enter separating gels.

VII. Cleavage of ADP–Ribose from Gα Subunits

Solutions containing the biological material, e.g., cell membranes or crude or purified proteins supplemented with 1 mg/ml BSA, should be buffered at a pH of 7.5. Samples are kept in glass tubes and treated simultaneously with 1 mM $HgCl_2$ or water (60 min, 37°C). The reaction is followed by precipitations with acetone/NaCl, TCA and chloroform/methanol. Analysis is usually done by SDS-PAGE.

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CHAPTER 10

Clostridium Botulinum C3 Exoenzyme and C3-Like Transferases

K. Aktories, H. Barth, and I. Just

A. Introduction

Clostridium botulinum adenosine diphosphate (ADP)–ribosyltransferase C3 was the first bacterial exoenzyme/toxin found to act on small guanosine triphosphate (GTP)ases of the Rho GTPase family. Subsequently, it has been realised that C3 is the prototype of a family of related ADP–ribosyltransferases (C3-like transferases) produced by a diverse group of bacteria, such as *C. botulinum*, *C. limosum*, *Bacillus cereus* and *Staphylococcus aureus*. Whereas the role of all these agents as virulence factors is still unclear, their importance as pharmacological tools is beyond question. Because C3-like exoenzymes specifically inactive Rho proteins, they are essential for the elucidation of the cellular functions of Rho GTPases. Here, we will review our current knowledge about *C. botulinum* C3 transferase and other C3-like transferases and will describe their origins, structures and functions. Moreover, another topic of this chapter will be the application of C3 as a pharmacological and cell-biological tool. Other bacterial agents targeting Rho GTPases are the large clostridial cytotoxins and the group of deamidating toxins from *Escherichia coli* and *Bordetella* species, which are discussed in detail in other chapters of this volume.

B. Origin and Purification of C3 Exoenzyme

I. Origin of C3 Exoenzyme

C3 was serendipitously discovered by screening for higher-producing strains of the actin ADP–ribosylating *C. botulinum* C2 toxin (Aktories et al. 1987). The exoenzyme is produced by various strains of *C. botulinum* types C and D, but not by other types (Fujii et al. 1988; Nemoto et al. 1991; Popoff et al. 1991; Hauser et al. 1993; Moriishi et al. 1993). Thus, C3-producing *C. botulinum* strains also synthesise botulinum neurotoxins (C1 and D) and the actin-ADP–ribosylating C2 toxin. Initially, co-expression of C3 together with botulinum neurotoxins caused some confusion, because it was suggested that the neurotoxins possess C3 activity. However, further purification of the toxins and precise immunological, biochemical and cell biological analyses revealed that C3 exoenzyme was a contaminant in many neurotoxin-C1 and -D prepara-

tions (Aktories and Frevert 1987; Adam-Vizi et al. 1988; Aktories et al. 1988b; Morii et al. 1990). Subsequent cloning and sequencing of the gene of C3 exoenzyme (see below) definitively proved that neurotoxins and C3 are separate, unrelated proteins. Later, this view was further verified by the finding that *C. limosum* produces a C3-like enzyme in the absence of any neurotoxin. Moreover, C3-like transferases were identified in the culture supernatants of *S. aureus* and *B. cereus*.

II. Purification of C3 Exoenzyme

All C3-like tranferases are 23-kDa to 28-kDa proteins with high contents of basic amino acids (pI = 9–10). Therefore, C3-like enzymes can be effectively purified by taking advantage of their basic isoelectric points. Originally, C3 was purified from a 48-h culture supernatant of the *C. botulinum* type-C strain Stockholm by ammonium sulfate (70%) precipitation, desalting on a Sephadex G-25 column and subsequent diethylaminoethyl Sephadex A-50 anion-exchange chromatography. The basic C3 recovered in the flow-through is finally purified to apparent homogeneity by gel filtration on Superdex 75 (Aktories et al. 1988b). A similar purification procedure is used for C3 from *C. limosum* (Just et al. 1992). Nemoto et al. (1991) purified C3 by using ammonium-sulfate precipitation, CM-Sephadex chromatography, gel filtration on a G-75 column and chromatography on Mono S HR column. For purification of C3, Moriishi et al. (1991) used ammonium-sulfate precipitation, gel filtration on Sephadex G-100 and hydroxylapatite column chromatography. The C3-like transferase from *S. aureus* was purified from the concentrated culture filtrate by cation exchange (SP-Toyopearl 650), hydroxylapatite chromatography (TSK HA-1000) and subsequent reverse-phase high-performance liquid chromatography using a column of VIDAC RP-C4 (Sugai et al. 1990).

Today, C3 exoenzymes are mainly produced as recombinant proteins lacking the signal sequence. Narumiya et al. use a modified enzyme that has Met–Ala attached to Ser1 of the mature enzyme; the modified gene is cloned into pET3a vector and is expressed under the control of the bacteriophage T7 promoter. Purification is performed from the *E. coli* lysate by CM-Sepharose and subsequent gel filtration (TSK-gel 3000SW; Saito and Narumiya 1997). Quite often, C3 is expressed as a glutathione *S*-transferase (GST) fusion protein and purified by affinity chromatography with glutathione–agarose beads. Subsequently, C3 is released from the agarose-bound fusion protein by thrombin cleavage (Nobes and Hall 1997). Both purification methods result in 1–3 mg of recombinant C3 from 1 l of culture. C3 from *C. botulinum* and *C. limosum* are very stable proteins and are active for months when stored at 4°C. C3 from *C. botulinum* type-C strain Stockholm is resistant towards short-term heating (1 min, 95°C) and somewhat resistant towards trypsin treatment (Aktories et al. 1987).

C. Genetics of C3 Exoenzyme

First, Popoff and coworkers showed that the C3 exoenzyme gene is located on a bacteriophage in *C. botulinum* types C (strain 468) and D (strain 1873; Popoff et al. 1990). These phages also encode for the respective botulinum neurotoxins C and D, which are 54% identical to each other at the amino acid level. By contrast, the C3 gene encoded by these phages is 100% identical. The open reading frame of the C3 gene covers 759 bp, and the deduced polypeptide is composed of 251 amino acid residues and has a predicted mass of 27,823 Da (Fig. 1). The mature protein covers Ala41–Lys251 (here designated "C3P"; predicted molecular mass 23 546 Da). The 40 N-terminal amino acids of the premature protein correspond to a signal peptide and show the general features of secretory signal peptides, with positively charged amino acids at the extreme N-terminus, a central hydrophobic core, a polar C-terminal region and a proteolytic-cleavage site (Lys40–Ala41; Popoff et al. 1991). It was assumed that the C3 gene is located on a mobile genetic element. However, the suggested mobile element did not display sequence similarity to known transposons or insertion sequences (Hauser et al. 1993). Typically, for clostridia genes, the A + T content of the C3 gene is rather high (by about 70%). Narumiya and coworkers cloned C3 by using total DNA from *C. botulinum* type-C strain 003-9 (Nemoto et al. 1991). Sequence analysis of the C3 gene revealed an open reading frame of 732 bp encoding a protein of 244 amino acids with a molecular mass of 27 362 Da. Again, a putative signal sequence of 40 amino acid residues preceded the mature protein of 204 residues (here designated C3N), which had a predicted molecular mass of 23 119 Da. Moriishi and coworkers cloned and sequenced several C3-like

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C3N      1 SYADTFTEFTNVEEA KKWGNAQYKKYGLSK PEQEAIKFYTRDASK INGPLRANQGNENGL  60
Clim     1 PYADSFKEFTNIDEA RAWGDKQFAKYKLSS SEKNALTIYTRNAAR INGPLRANQGNTNGL  60
C3P      1 AYSNTYQEFTNIDQA KAWGNAQYKKYGLSK SEKEAIVSYTKSASE INGKLRQNKGVINGF  60
EDIN     1 --AD-VKNFTDLDEA TKWGNKLIKQAKYSS DDKIALYEYTKDSSK INGPLRLAGGDINKL  57

C3N     61 PADILQKVKLIDQSF SKMKMPQNIILFRGD DPAYLG--PEFQDKI LNK-----DGTINKT 113
Clim    61 PADIRKEVEQIDKSF TKMQTPENIILFRGD DPGYLG--PDFENTI LNR-----DGTINKA 113
C3P     61 PSNLIKQVELLDKSF NKMKTPENIMLFRGD DPAYLG--TEFQNTL LNS-----NGTINKT 113
EDIN    58 DSTTQDKVRRLDSSI SKSTTPESVYVYRLL NLDYLTSIVGFTNED LYKLQQTNNGQYDEN 117

C3N    114 VFEQVKAKFLKKDRT EYGYISTSLMS-AQF GGRPIVTKFKVTNGS KGGYIDP--ISYFPG 170
Clim   114 VFEQVKLRFKGKDRK EYGYISTSLVNGSAF AGRPIITKFKVLDGS KAGYIEP--ISTFKG 171
C3P    114 AFEKAKAKFLNKDRL EYGYISTSLMNVSQF AGRPIITKFKVAKGS KAGYIDP--ISAFAG 171
EDIN   118 LVRKLNNVMNSRIYR EDGYSSTQLVSGAAV GGRPIELRLELPKGT KAAYLNSKDLTAYYG 177

C3N    171 QLEVLLPRNNSYYIS DMQISPNNRQIMITA MIFK------                      204
Clim   172 QLEVLLPRSSTYTIS DMQIAPNNKQIIITA LLKR------                      205
C3P    172 QLEMLLPRHSTYHID DMRLSSDGKQIIITA TMMGTAINPK                      211
EDIN   178 QQEVLLPRGTEYAVG SVELSNDKKKIIITA IVFKK-----                      212
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Fig. 1. Alignment of the amino acid sequences of C3-like exoenzymes. *C3N*, C3 exoenzyme from *Clostridium botulinum* type C from strain C-003-9 [accession number (Acc. no.) Q00901]; *C3P*, C3 exoenzyme from *C. botulinum* type C strain C468 (Acc. no. X59039); *C3lim*, C3 exoenzyme from *C. limosum* (Acc. no. Q46143); *EDIN*, C3-like exoenzyme from *Staphylococcus aureus* (Acc. no. P24121)

transferases from *C. botulinum* type D and also identified two major isoforms of C3 largely resembling C3P and C3N (Moriishi et al. 1993).

The gene for a C3-like ADP-ribosyltransferase was cloned from *C. limosum* total DNA. It contained an open reading frame of 750 bp encoding for a protein of 250 amino acid residues with a molecular mass of 27 840 Da. The encoded protein consists of a signal sequence of 45 amino acid residues, and the mature protein starts after Lys45 with Pro46. The nucleotide sequence of *C. limosum* transferase was identical to the sequences of C3 ADP-ribosyltransferases reported by Popoff et al. (1990) and Nemoto et al. (1991) by about 71% and 70%, respectively. At the amino acid level, *C. limosum* transferase was identical to the C3 isoforms from *C. botulinum* by about 60% and 65%, respectively.

However, C3-like transferases are not only restricted to clostridia. Inoue et al. (1991) reported on the cloning and sequencing of a C3-like transferase from *S. aureus*, which was designated as EDIN (epidermal cell differentiation inhibitor). DNA sequencing revealed a 247 amino acid precursor protein including a 35 amino acid N-terminal signal sequence. The mature EDIN protein is composed of 212 amino acid residues with a calculated mass of 23 782 Da and shares about 35% identity with C3 exoenzymes from *C. botulinum*. Another C3-like transferase has been identified in *B. cereus* (Just et al. 1995a). This enzyme has not been cloned. Amino acid sequences available from direct protein microsequencing indicate that this enzyme is more distantly related to *C. botulinum* C3 transferase.

D. Structure–Function Analysis of C3 Exoenzyme

All C3-like exoenzymes are mono-ADP-ribosyltransferases like other well-known bacterial toxins, such as cholera toxin, pertussis toxin and diphtheria toxin (DT; Domenighini and Rappuoli 1996; see respective chapters for details). These agents were originally called A–B toxins, because they consist of an active enzyme component (A) and a binding component (B). More recent structure analysis of bacterial protein toxins, which is based on the crystal structures of the proteins, revealed a tripartite structure of most toxins. The toxins had three distinct domains: one for membrane receptor binding, a second for toxin translocation into the cytosol and a third, catalytically active domain for covalent modification of the target protein (Collier 1995; Hol et al. 1995). C3 exoenzymes, however, differ from these toxins, because they consist of the catalytic domain only.

During recent years, major progress was made in the structure–function analysis of mono-ADP-ribosyltransferases (Chap. 2), which was mainly based on the availability of crystal structures of various ADP-ribosylating toxins (Sixma et al. 1991; Choe et al. 1992; Stein et al. 1994; Weiss et al. 1995; Bell and Eisenberg 1996). These studies revealed that the overall structures of bacterial ADP-ribosyltransferases are very similar (Domenighini et al. 1995).

Moreover, bacterial ADP–ribosylating toxins share significant structural homology with eukaryotic transferases. However, this homology is almost entirely reflected in the three-dimensional structure of the ADP–ribosyltransferases, the few essential amino acid residues conserved most likely involved in nicotinamide adenine dinucleotide (NAD) binding and catalysis. At least three regions can be identified in most ADP–ribosyltransferases (Fig. 2). Region 1 contains an arginine residue (histidine in DT) and is suggested to be involved in NAD binding. About 50–80 residues downstream is located an STS motif (serine–threonine–serine) and (again, about 50–80 amino acid residues downstream) a so-called catalytic glutamic acid residue, which is strictly conserved in all ADP–ribosyltransferases known (Chap. 2). These regions are also highly conserved in C3-like exoenzymes. The arginine residue of region 1 most likely resembles Arg88 in C3 from *C. botulinum/limosum*. The STS motif is located at position 134 in C3. Finally, in C3P from *C. botulinum* (Glu173 in C3N) and in C3 from *C. limosum*, Glu174 cor-

C3 P	84	IMLF**R**GDDP ...	129	EYGYI**STS**LMNV ...	168	AFAG**Q**L**E**MLLP
C3 N	84	IILF**R**GDDP ...	129	EYGYI**STS**LMSA ...	167	YFPG**Q**L**E**VLLP
C3 Lim.	84	IILF**R**GDDP ...	129	EYGYI**STS**LVNG ...	168	TFKG**Q**L**E**VLLP
EDIN	81	VYVY**R**LLNL ...	133	EDGYS**ST**QLVSG ...	174	AYYG**QQE**VLLP
PT	5	ATVY**R**YDSR ...	47	NSAFV**STS**SSRR ...	123	LATY**Q**S**E**YLAH
CT	3	DKLY**R**ADSR ...	56	DDGYV**STS**ISLR ...	106	PHPD**EQE**VSAL
C2	295	LIAY**R**RVDG ...	342	NLSFS**STS**LKST ...	383	GFQD**EQE**ILLN
Iota	291	LIVY**R**RVDG ...	333	YPNFI**STS**IGSV ...	374	GYAG**E**Y**E**VLLN
ExoS			351	GFTYL**STS**LNPG ...	388	NYKN**EKE**ILYN
RT6.1	142	HSVY**R**GTKT ...	162	FGQFT**SSS**LSKT ...	203	FYPD**QE**EVLIP

Fig. 2. Alignment of conserved regions of adenosine diphosphate (ADP)–ribosyltransferases. The arginine residue from the first region might be involved in nicotinamide adenine diphosphate (NAD) binding and in stabilisation of the integrity of the active site (Bell and Eisenberg 1996). The STS motif from the second region is suggested to be involved in NAD binding (nicotinamide mononucleotide portion). The third region contains the conserved catalytic glutamic acid residue. In some transferases, the preceding glutamic acid residue (in C2 toxin) is essential for transferase but not for glucohydrolase activity. *C3P*, C3 exoenzyme from *Clostridium botulinum* type C strain C468 [accession number (Acc. no.) X59039]; *C3N*, C3 exoenzyme from *C. botulinum* type C from strain C-003-9 (Acc. no. Q00901); *C3lim*, C3 exoenzyme from *C. limosum* (Acc. no. Q46143); EDIN, C3-like exoenzyme from *Staphylococcus aureus* (Acc. no. P24121); *PT*, pertussis toxin, enzymatic subunit S1 (Acc. no. M13223); *CT*, cholera toxin, enzymatic subunit A (Acc. no. P01555); *C2*, *C. botulinum* C2 toxin enzymatic component C2I (Acc. no. AJ224480); *IOTA*, *C. perfringens* iota toxin, enzymatic component A (Acc. no. X73562); *ExoS*, *Pseudomonas* exoenzyme S (Acc. no. L27629); *RT6.1*, mammalian mono-ADP–ribosyltransferase in rat (Acc. no. X252082)

responds to the catalytic glutamic residue. The essential role of Glu174 for enzyme activity was verified by mutagenesis of C3 from *C. limosum* (Gesellchen et al., unpublished); an essential role was also verified for Glu173 in C3N (Saito et al. 1995). Similarly, as observed with the catalytic glutamic acid residues of *Pseudomonas* exotoxin A (Carroll and Collier 1987), DT (Carroll et al. 1985) and pertussis toxin (Barbieri et al. 1989), Glu174 of C3 from *C. limosum* was labelled by ultraviolet irradiation in the presence of [carbonyl-^{14}C]NAD (Jung et al. 1993). Moreover, the equivalent amino acid residue was radiolabelled with [carbonyl-^{14}C]NAD in the C3-like transferase from *B. cereus* (Just et al. 1995a). Thus, all data available suggest that ADP-ribosylation catalysed by C3 follows the same reaction mechanism as shown for other bacterial ADP-ribosylating toxins. However, C3-like transferases are unique in their acceptor amino acid residue of the protein target; they modify asparagine residues (see below).

E. ADP-Ribosyltransferase Activity

I. Basic Properties

Studies from recent years indicate that C3 and all C3-related exoenzymes catalyse the same mono-ADP-ribosylation of Rho proteins. Accordingly, phosphodiesterase treatment of Rho ADP-ribosylated by C3 releases 5′-adenosine monophosphate (AMP) and not phosphoribosyl AMP, a cleavage product of poly(ADP-ribose; Aktories et al. 1988b; Rubin et al. 1988). The reaction is specific for NAD and is not observed with ADP-ribose, which serves as a co-substrate for non-enzymatic ADP-ribosylation. ADP-ribosylation is not abolished by thymidine, which is an inhibitor of poly(ADP-ribose) polymerase, nor is it blocked by isonicotinic-acid hydrazide, an inhibitor of NAD glycohydrolases (Rubin et al. 1988). The K_m value for NAD was calculated to be approximately 0.4 μM (Just et al. 1992). However, for unknown reasons, the K_m of the recombinant *C. limosum* C3 was about 100 times higher with recombinant Rho than with native Rho (Böhmer et al. 1996). The specific activity was determined to be 6.4 nmol/min/mg (Just et al. 1992). As for other ADP-ribosyltransferases, C3 possesses NAD-glycohydrolase activity (Aktories et al. 1988b). However, this activity is very low. The pH optima for ADP-ribosylation and the reverse reaction (de-ADP-ribosylation in the presence of nicotinamide and the absence of NAD) are 7.5 and 5.5, respectively (Habermann et al. 1991).

II. Regulation by Detergents, Nucleotides and Divalent Cations

ADP-ribosylation of Rho proteins is affected by various lipids and detergents (Williamson et al. 1990; Bourmeyster et al. 1992; Just et al. 1992, 1993). Whereas sodium dodecyl sulfate (SDS; 0.01%), sodium cholate (0.2%), dimyristoylphosphatidylcholine (3 mM) and desoxycholate (0.1%) increase C3 ADP-ribosylation (Williamson et al. 1990; Just et al. 1993), SDS

(>0.03%), 3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate, Lubrol-PX and various phospholipids decrease ADP–ribosylation (Just et al. 1993). Also, amphiphatic agents like mastoparan, mellitin and compound 48/80 inhibit ADP–ribosylation of Rho by C3 (Koch et al. 1992). The effects of detergents on C3-catalysed ADP–ribosylation depend on the specific Rho preparation. Stimulation of Rho labelling was observed with human platelet cytosol, recombinant RhoA and RhoA purified from bovine brain cytosol (Williamson et al. 1990; Just et al. 1992, 1993). By contrast, human platelet-membrane Rho, recombinant RhoB, and RhoB purified from bovine brain cytosol were not affected (Williamson et al. 1990; Just et al. 1992, 1993). Most likely, the effect of SDS on ADP–ribosylation occurs on Rho, because the detergent has no stimulatory effect on the NAD-glycohydrolase activity of C3. Two mechanisms are conceivable: first, the detergents or certain phospholipids (phosphatidylinositol phosphates) split Rho-protein complexes [Rho–guanosine diphosphate (GDP)-dissociation inhibitor (GDI) complex], which are poor substrates for ADP–ribosylation (Bourmeyster et al. 1992; Kikuchi et al. 1992). Second, the detergents (SDS at low concentration) may provide a more lipophilic environment for the GTPase thereby favouring the ADP–ribosylation. In this respect, it is noteworthy that, in the presence of SDS (0.01%), the ability of Rac to serve as a substrate for C3 (from *C. botulinum* but not from *C. limosum*) is increased (up to 10% of ADP–ribosylated Rac is detected; Just et al. 1992).

ADP–ribosylation of Rho proteins by C3 is regulated by magnesium ions and guanine nucleotides. C3-catalysed ADP–ribosylation of cytosolic Rho proteins is enhanced by magnesium, calcium, barium and manganese ions; cadmium and lanthanum (each 5 mM) reduce it (Narumiya et al. 1988). Magnesium exhibits a biphasic curve, with a stimulatory effect up to 10 mM, whereas higher concentrations inhibit ADP–ribosylation. The influence of guanine nucleotides on C3-induced ADP–ribosylation depends on the assay conditions and Rho preparation used. In the absence of magnesium ions or in the presence of chelators (ethylene diamine tetraacetic acid), guanine nucleotides have stimulatory effects. In the absence of the divalent cation, the nucleotide is released and the protein is likely to be degraded (Habermann et al. 1991). In the presence of magnesium, ADP–ribosylation of membrane-bound Rho or recombinant RhoA is lower with GTP or GTPγS than with GDP (Habermann et al. 1991). However, cytosolic Rho is a better substrate for C3 with GTP or GTPγS than with GDP (Williamson et al. 1990). Most likely, GTP and GTPγS favour the dissociation of the Rho–GDI complex, thereby increasing the ADP–ribosylation of Rho.

III. Rho Proteins as Substrates for C3

The Rho subfamily of GTPases is comprised of more than ten members, which can be listed in three groups. The first group is composed of Rhos, including RhoA, RhoB, RhoC, RhoD, RhoE and TTF. The second group includes four Rac proteins (Rac 1–3 and RhoG), and the third group consists of Cdc42,

G25K and TC10. C3 ADP-ribosylates RhoA, B and C (AKTORIES et al. 1989; BRAUN et al. 1989; CHARDIN et al. 1989; Fig. 3). The modification of Rho occurs at Asn41 (SEKINE et al. 1989; Fig. 4). Although most other members of the Rho GTPase family contain an equivalent asparagine residue, they are poor in vitro substrates (Rac, see above) or are not at all modified (RhoE; NOBES et al. 1998). No evidence exists that Rho GTPases (other than RhoA, B and C) are modified by C3 in intact cells. ADP-ribosylation of asparagine is unique to C3-like transferases. Other ADP-ribosylating toxins modify arginine residues (cholera toxin, *E.coli* heat-labile toxins, *C. botulinum* C2 toxin, *Pseudomonas* exoenzyme S), cysteine residues (pertussis toxin) or diphthamide, a post-

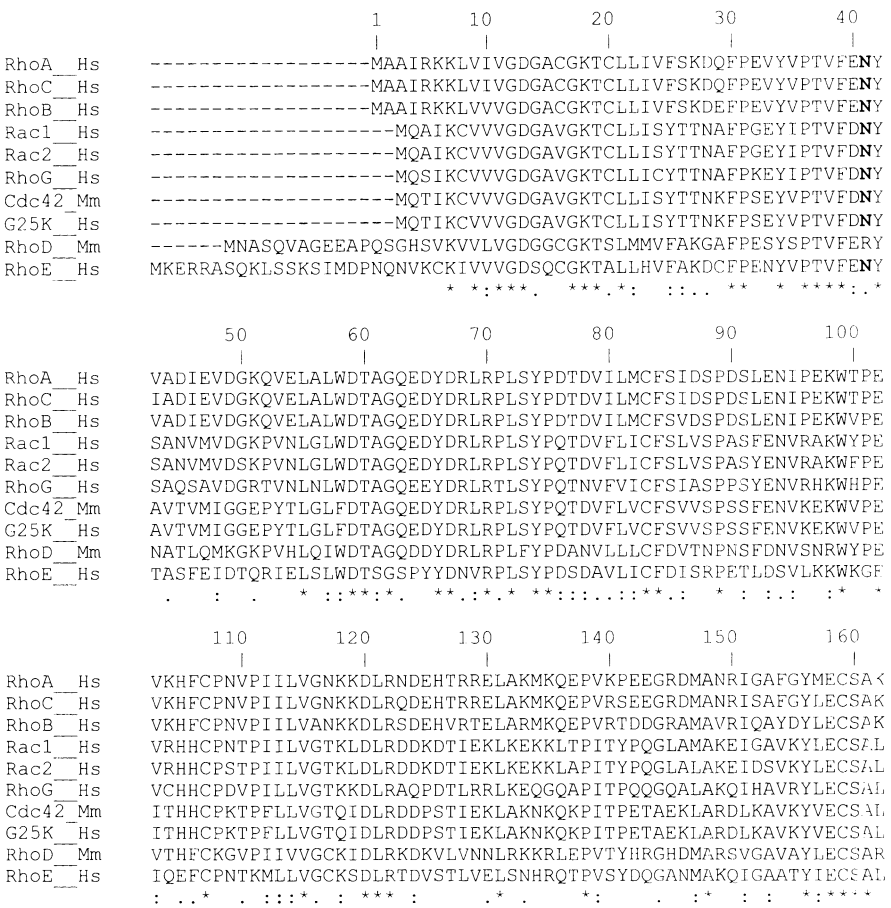

Fig. 3. Alignments of Rho guanosine triphosphate (GTP)ases. Numbering corresponds to RhoA. Asparagine-41 of RhoA and the respective asparagine residues of other Rho GTPases are in *bold letters*. Swissprot accession numbers: RhoA_Hs (P06749), RhoC_Hs (P08134), RhoB_Hs (P01121), Rac1_Hs (P15154), Rac2_Hs (P15153), RhoG_Hs (P35238), Cdc42_Mm (P25763), G25K_Hs (P21181), RhoD_Mm (P97348), RhoE_Hs (P52199)

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                 170        180        190        200        210        220
                  |          |          |          |          |          |
RhoA__Hs     -TKDGVREVFEMATRAALQARRGKKK-------------------------------SG
RhoC__Hs     -TKEGVREVFEMATRAGLQVRKNKRR-------------------------------RG
RhoB__Hs     -TKEGVREVFETATRAALQKRYGSQNG-----------------------------CINC
Rac1__Hs     -TQRGLKTVFDEAIRAVLCPPPVKK-------------------------------RKRK
Rac2__Hs     -TQRGLKTVFDEAIRAVLCPQPTRQ-------------------------------QKRA
RhoG__Hs     -QQDGVKEVFAEAVRAVLNPTPIK--------------------------------RGRS
Cdc42_Mm     -TQKGLKNVFDEAILAALEPPEPK--------------------------------KSRR
G25K__Hs     -TQRGLKNVFDEAILAALEPPETQ--------------------------------PKRK
RhoD__Mm     -LHDNVEAVFQEAAEVALSSRRHNFWRR----------------------------ITQN
RhoE__Hs     QSENSVRDIFHVATLACVNKTNKNVKRNKSQRATKRISHMPSRPELSAVATDLRKDKAKS
               . .:. :*  *  . :

RhoA__Hs     CLVL-
RhoC__Hs     CPIL-
RhoB__Hs     CKVL-
Rac1__Hs     CLLL-
Rac2__Hs     CSLL-
RhoG__Hs     CILL-
Cdc42_Mm     CVLL-
G25K__Hs     CCIF-
RhoD__Mm     CCLAT
RhoE__Hs     CTVM-
             * :
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Fig. 3. *Continued*

translationally modified histidine residue (DT, *Pseudomonas* exotoxin A). Whereas the arginine–ADP–ribose linkage is cleaved by hydroxylamine (0.5 M, 2 h), and $HgCl_2$ (1 mM, 30 min) splits the pertussis-toxin-formed bond of ADP–ribose with cysteine, the asparagine–ADP–ribose linkage is stable towards both treatments (Aktories et al. 1988a).

Although C3 ADP–ribosylates exclusively Rho A, the transferase appears to interact with Ral, a GTPase belonging to the Ras subfamily. Addition of Ral to cell lysate blocks the ADP–ribosylation of Rho by C3 (Rex, Just and Aktories, unpublished observation). Similarly, C3 inhibits the glucosylation of Ral by *C. sordellii* lethal toxin. Moreover, a direct interaction of Ral with C3 was verified by precipitation of the transferase by GST–Ral Sepharose beads. The relevance of this interaction is not clear.

IV. Functional Consequences of the ADP–Ribosylation of Rho

Asn41 is located on the extended β-strand B2 and near the so-called switch-1 region (residues 28–38) of the GTPase. The switch-1 region and its C-terminal flanking regions undergo major conformational changes, depending on the nucleotide bound, thereby playing a key role as a molecular switch in signal transduction. The side chain of Asn41 forms a hydrogen bond (3.1 Å) to the main-chain carbonyl group of Glu40 (Ihara et al. 1998). This allows Asn41 to interact with the indole ring of Trp58 of strand B3. Since the indole ring functions as a strong electron donor, this interaction enhances the nucleophilicity of the side-chain nitrogen of Asn41 (Ihara et al. 1998). Because

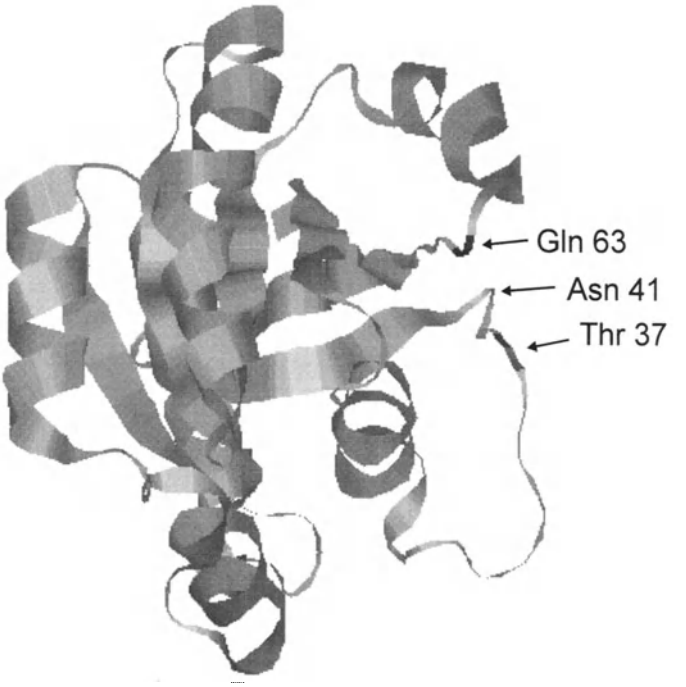

Fig. 4. Crystal structure of Rho. Asparagine 41 is adenosine diphosphate–ribosylated by C3-like transferases. Threonine 37 is glucosylated by large clostridial cytotoxins. Glutamine-63 is deamidated by cytotoxic necrotising factors CNF1 and CNF2 from *Escherichia coli* and by the dermonecrotic toxin from *Bordetella* (crystal-structure data are from WEI et al. 1997)

mono-ADP–ribosylation reactions are suggested to occur by a nucleophilic attack (by the protein substrate) on an oxocarbonium-like transition state of the ribose moiety of the ADP–ribose, increase in nucleophilicity will favour the ADP–ribosylation of Asn41 (Locht and Antoine 1997).

The side chains of Asn41 are oriented away from the effector loop (IHARA et al. 1998). Accordingly, it was reported that ADP–ribosylation has only minor effects on the nucleotide binding and GTPase activity of Rho. ADP-ribosylation of Rho slightly increased the release of GDP (control RhoA: 0.047/min; ADP–ribosylated: k_{GDP} = 0.056/min) and decreased the release of GTPγS (control RhoA: $k_{GTP\gamma S}$ = 0.013/min; ADP–ribosylated RhoA: k_{GTP} = 0.007/min; SEHR et al. 1998). The intrinsic GTPase activity was increased from 0.027/min (control) to 0.060/min (ADP–ribosylated RhoA). Stimulation of GTPase activity by Rho–GTPase-activating protein (GAP) was eight- and fivefold with control and ADP–ribosylated Rho, respectively. Surprisingly, ADP–ribosylated Rho is still able to interact with its effectors (protein kinase N; SEHR et al. 1998). Thus, the blockade of the Rho/effector interaction is not the molecular mechanism underlying the biological inactivation of Rho by ADP–ribosylation. In contrast, it has been reported that ADP–ribosylation of

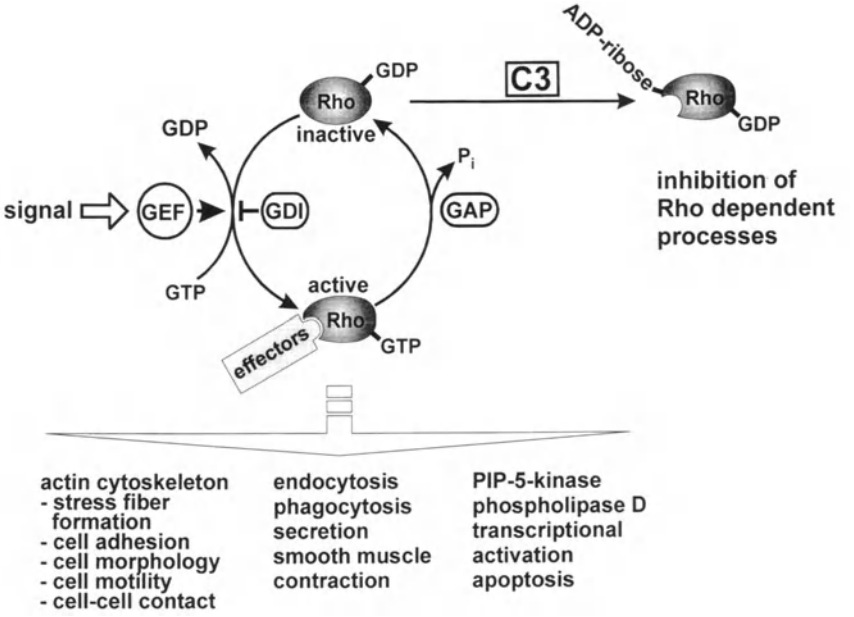

Fig. 5. Rho proteins act as molecular switches. Rho proteins are regulated by a guanosine triphosphate (GTP)ase cycle. The GTPases are inactive with guanosine diphosphate (GDP) bound. Nucleotide exchange of GDP with GTP, induced by guanine-nucleotide-exchange-factor proteins, activates Rho. Guanine-nucleotide-dissociation inhibitors block the exchange. In the GTP-bound form, Rho proteins (Rho, Rac, and Cdc42) interact with a large array of effectors to elicit the cellular effects as indicated. The active state of Rho GTPases is terminated by hydrolysis of GTP. This hydrolysis is stimulated by GTPase-activating proteins. C3 ADP–ribosylates Rho (RhoA, B and C, but not Rac or Cdc42) at asparagine 41. ADP–ribosylated Rho is biologically inactive. The precise mechanism of the inactivation is not clear (see text for details). Effectors are still able to bind to ADP–ribosylated Rho

Rho increases its affinity for its effector. ADP–ribosylated Rho–GST beads showed an approximately eightfold-higher binding to phosphatidylinositol-4-phosphate-5 (PIP5) kinase than that shown by control Rho. Accordingly, a higher salt concentration (1000 mM NaCl versus 250 mM) was necessary to release the PIP5 kinase from ADP–ribosylated Rho, indicating a higher affinity of the modified Rho for the kinase (Ren et al. 1996). These authors suggested that C3 has a dominant negative effect by sequestering the effector without activating it. Consistent with this hypothesis are findings that microinjection of ADP–ribosylated Rho into Swiss 3T3 cells induces the typical C3 effects in a dominant manner (Paterson et al. 1990; Ridley and Hall 1992). Moreover, co-injection of C3 with the C3-resistant mutant N41L RhoA was not able to prevent the typical C3 phenotype (Paterson et al. 1990). However, several results are not compatible with a dominant inhibitory effect of ADP–ribosylated Rho (Fig. 5).

Co-expression of C3 and C3-resistant N41I RhoA in NIH 3T3 cells restored the RhoA-dependent pathway to activate transcription. This pathway was blocked by expression of C3 alone (Hill et al. 1995). Thus, endogenous Rho ADP-ribosylated by C3 does not appear to be dominant negative under these conditions. In line with the latter findings are recent studies in Hela cells. Narumiya and coworkers inactivated endogenous Rho by transfection of the C3 gene and then studied the activity of Rho chimeras by microinjection (Fujisawa et al. 1998). Again, C3-induced ADP-ribosylation of endogenous Rho apparently did not cause dominant negative effects. At present, the reason for these discrepancies are not clear but may depend on the cell type studied. What are alternative mechanisms by which ADP-ribosylation renders Rho inactive? Preliminary results from our laboratory indicate that the ADP-ribosylation largely reduces the activation of Rho by exchange factors (Sehr et. al., unpublished) and prevents the membrane translocation of Rho usually observed during the activation of Rho (Genth et al., unpublished). Both effects may have major impacts on the inhibition of the biological activity of Rho by ADP-ribosylation.

Although ADP-ribosylation does not appear to inhibit the interaction of the GTPase with certain effectors, the modification at Asn41 prevents subsequent glucosylation of Rho by large clostridial toxins, which occurs at Thr37. Vice versa, glucosylation of Rho by these toxins at Thr37 inhibits subsequent ADP-ribosylation at Asn41 by C3 (Just et al. 1994).

F. Application of C3-Like Exoenzymes as Tools

As mentioned above, C3 consists only of the catalytic domain but appears to possess no specific receptor-and-translocation domain. Therefore, the uptake of C3 into cells is generally poor. C3 is supposed to enter cells by non-specific pinocytosis. However, the precise mechanism, including translocation into the cytosol, is largely obscure. Because C3 is characterised by a rather poor cell accessibility, it has to be applied at high concentrations (Wiegers et al. 1991; Morii and Narumiya 1995; Amano et al. 1996; Verschueren et al. 1997). Usually, 5–50 μg/ml of the toxin is used in cell cultures, and the incubation time is often extended to up to 24–48 h. Lipofectamine is used to facilitate the uptake (Hirao et al. 1996; Renshaw et al. 1996). In most studies, C3 has been introduced into cells by microinjection (Paterson et al. 1990; Ridley and Hall 1992, 1994; Chong et al. 1994; Watanabe et al. 1997; Olson et al. 1998). In fact, numerous studies have been reported in which microinjection of C3 was the major tool used to alter Rho functions in intact cells. The disadvantage of this method is the small number of cells, which does not allow biochemical study of the cells treated. Therefore, other approaches to introduce C3 into culture cells have been successfully employed, such as permeabilization of cells by digitonin (Mackay et al. 1997), streptolysin O (Fensome et al. 1998), electrical discharge (electropermeabilization; Stasia et al. 1991; Koch et al. 1994) or by scrape loading (Barry et al. 1997). Another approach is the transfection of the C3 gene into eukaryotic cells. This is usually carried out by transient transfec-

tion (Hill et al. 1995; Caron and Hall 1998; Fujisawa et al. 1998). An exciting approach is the stable transfection of C3 by using a thymus-specific expression vector based on the proximal promoter of $p56^{lck}$ (Henning et al. 1997). By this means, transgenic mice exhibiting ADP–ribosylation of Rho specifically in the thymus were generated.

To circumvent the poor cell accessibility of C3, toxin chimeras consisting of the cell-binding and membrane-translocation domains of complete toxins and having C3 as the enzyme component have been constructed (Figs. 6, 7). For this purpose, C3 was fused to the binding-and-translocation subunit of DT. Interestingly, the fusion protein DC3B was most effective when 17 amino acid residues (which cover the disulfide bridge of the toxin) from the C-terminus of the A component of DT were included in the fusion construct (Aullo et al. 1993; Nusrat et al. 1995; Vouret-Craviari et al. 1998). With the chimeric toxin DC3B, 600 pg/ml was sufficient to induce the redistribution of F-actin structures in Vero cells. Although the fusion toxin is efficiently transported into cells, the underlying translocation pathways and mechanisms may differ from that of native DT, because the fusion toxin was not able to translocate into the cytosol upon acidification (Aullo et al. 1993). Recently, the *C. botulinum* C2 toxin was used to construct a chimeric C3-fusion toxin. C2 toxin is binary in structure and consists of the actin–ADP–ribosylating enzyme component C2I and the binding-and-translocation component C2II (Ohishi et al. 1980; Aktories et al. 1986; Aktories and Wegner 1989). Both components are separate proteins which act together after receptor binding of C2II at the surface of the target cell (Chap. 18). The N-terminal part of the binary enzyme component (C2I), which interacts with the binding component C2II, was fused to full-length C3. The fusion toxin was efficiently taken up by Chinese-hamster-ovary and Hela cells, and the typical cytotoxic effects were observed at 200 ng/ml C2IN-C3 and 200 ng/ml C2II after 1–2 h of incubation (Fig. 7). In the cytosol, Rho – but not actin (the actin ADP–ribosylation activity was deleted) – was ADP–ribosylated by the fusion toxin (Barth et al. 1998).

G. Cellular Effects of C3 Exoenzymes

As stated above, C3-like transferases have a significant impact on the elucidation of functions of Rho GTPases. In the following paragraphs, examples of these studies, documenting the successful application of C3 as a pharmacological and cell biological tool, are reviewed. Excellent, comprehensive reviews on the functions of Rho GTPases have been published recently (Collard 1996; Lim et al. 1996; Narumiya 1996; Symons 1996).

I. Effects of C3 on Cell Morphology and Actin Structure

Depending on the cell type studied, treatment of intact cells with high concentrations of C3 or microinjection of C3 into culture cells causes major changes of the actin cytoskeleton. It was first shown by treatment of Vero cells that C3 (5 μg/ml, for 12–24 h) induces rounding up of cells, with concomitant

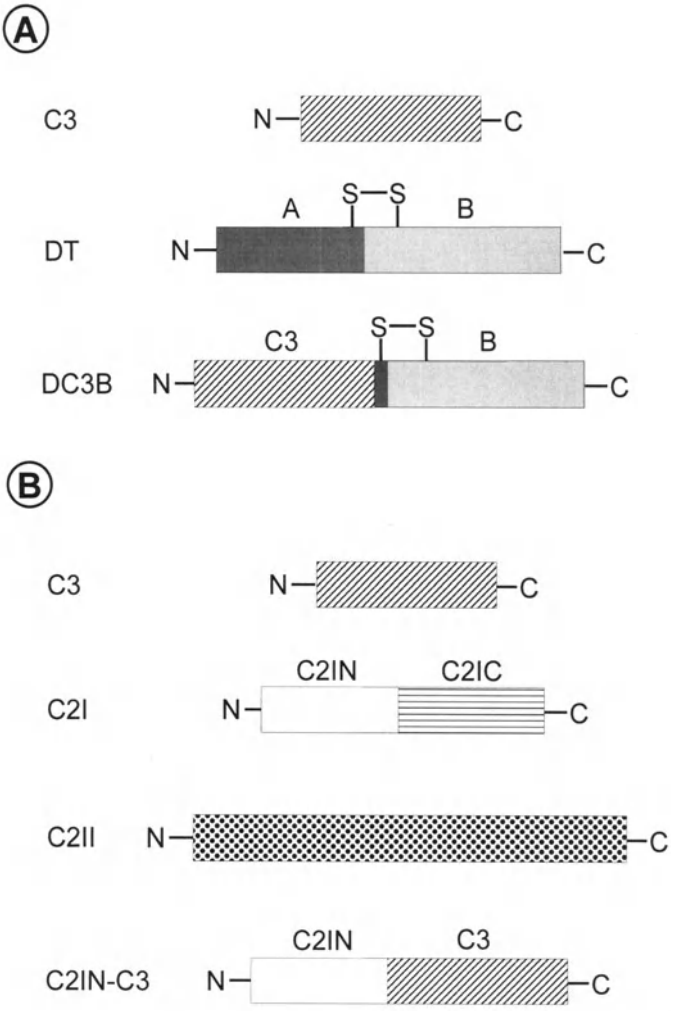

Fig. 6A,B. Chimeric C3 fusion toxins. To facilitate the cellular uptake of C3 exoenzyme, chimeric fusion toxins are constructed. **A** Fusion toxin DC3B on the basis of diphtheria toxin. The C-terminus-located binding component B of diphtheria toxin and 17 amino acid residues of the A component, covering the disulfide bridge of diphtheria toxin, are fused to C3 to form DC3B (AULLO et al. 1993). **B** Fusion toxin C2IN-C3 on the basis of C2 toxin. *Clostridium botulinum* C2 toxin consists of the enzyme component C2I and the cell-binding component C2II. C3 was fused to the N-terminal part of C2I, which interacts with C2II, to form C2IN-C3 (BARTH et al. 1998)

destruction of stress fibres (CHARDIN et al. 1989). These results were confirmed with various other cell types. Typically, the cells remain in contact via small extensions. In contrast to C3, treatment of cells with the actin ADP-ribosylating C2 toxin, which completely blocks actin polymerisation, causes complete rounding up, with loss of cell contacts (WIEGERS et al. 1991). After

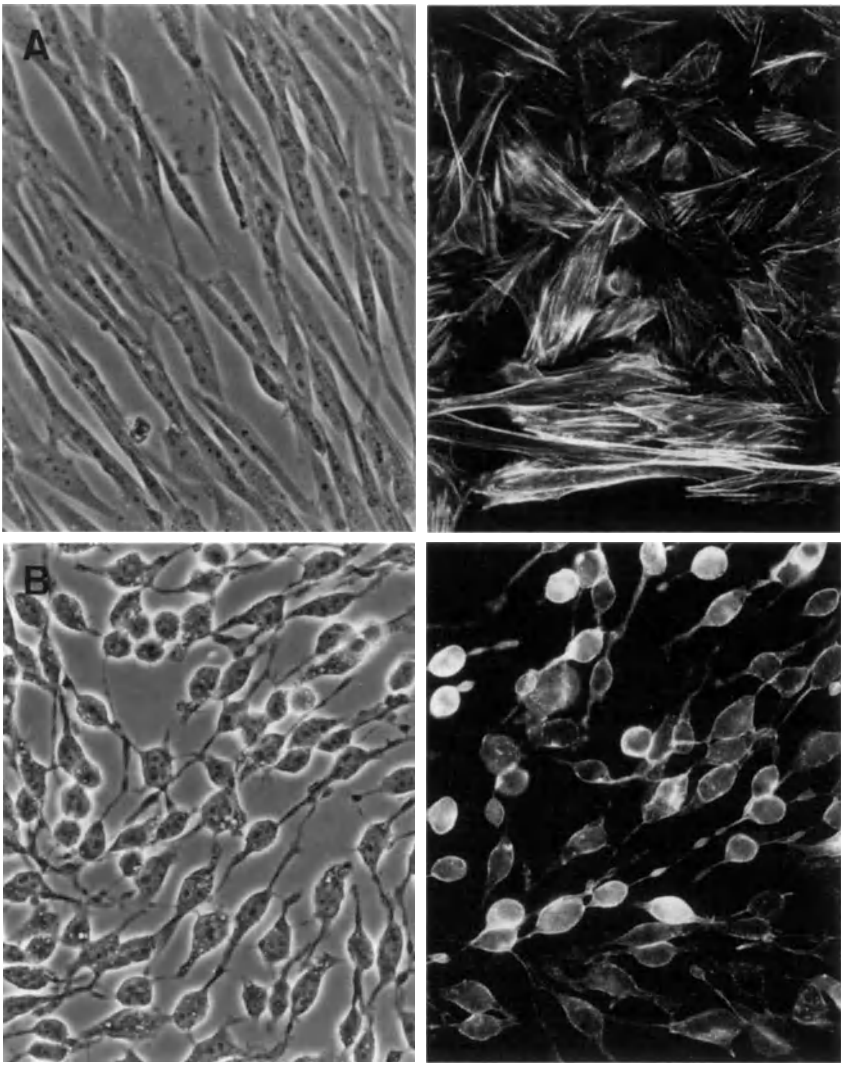

Fig. 7. Effects of the fusion toxin C2IN-C3 on culture cells. Chinese-hamster ovary cells were treated without (**A**) or with (**B**) the chimeric toxin C2IN-C3 (200 ng/ml) and the binding component C2II (200 ng/ml) for 3 h. Thereafter, cells were fixed for microscopy and actin-staining by phalloidin–rhodamine (*right panel*)

C3 treatment, cells are still viable, and change of the culture medium causes reversal of rounding after 1–2 days (Chardin et al. 1989; Wiegers et al. 1991). Actin staining by rhodamine–phalloidin reveals loss of stress fibres, and rhodamine–phalloidin-decorated F-actin is often concentrated into a few larger aggregates. Cortical actin is much more resistant towards C3 treatment. Most important for the understanding of the effects of C3 and for the elucidation

of the role of Rho proteins in the organisation of the actin cytoskeleton were microinjection studies (Paterson et al. 1990; Ridley and Hall 1992; Hall 1994). In these studies, rounding up and specific destruction of the actin cytoskeleton is observed earlier (10–15 min after microinjection) than after addition to the culture medium. By this means, it was demonstrated that C3-induced inactivation of Rho prevents growth-factor-induced formation of stress fibres and focal adhesions (Ridley and Hall 1992; Hall 1994; Mackay et al. 1997). Similarly, integrin-induced formation of stress fibres and focal adhesions is blocked by C3 in Swiss 3T3 cells (Barry et al. 1997). However, in fibroblasts, formation of lamellipodia and microspikes, which are under the control of Rac and Cdc42 GTPases, respectively, are not affected by C3, emphasising the specificity of Rho ADP–ribosylation (Ridley et al. 1992; Kozma et al. 1995; Nobes and Hall 1995). However, one has to keep in mind that the effects of C3 depend on the cell type studied. For example in KB cells, a cell line derived from epidermoid carcinoma, or in Madin-Darby canine kidney (MDCK) cells, membrane ruffling induced by hepatocyte growth factors or phorbol esters depends on Rho and is prevented by microinjection of C3 (Nishiyama et al. 1994). Although C3 induces rounding up in adherent cells like fibroblasts or Vero cells, in monocytes, C3 causes cell spreading (Aepfelbacher et al. 1996). Moreover, in the human myelomonocytic U937 cell line, C3 enhances adhesion to fibronectin via $\alpha 5\beta 1$-integrin receptors (Aepfelbacher 1995).

A surprising effect was observed with C3 in neuronal cells. Different groups showed that microinjection or addition of C3 to the culture medium causes inhibition of thrombin or lysophosphatidic acid (LPA)-induced neurite retraction and cell rounding in neuronal-like cells (Jalink et al. 1994; Tigyi et al. 1996a, 1996b; Katoh et al. 1998). It was suggested that LPA causes receptor-mediated contraction of these cells via a pathway involving Rho kinase/ROKα and myosin phosphorylation (Katoh et al. 1998). These studies with C3 were the starting point for the hypothesis that Rho-family GTPases play a role in neuritogenesis (Mackay et al. 1995).

C3 was applied to study the role of Rho in various motile functions of cells. The exoenzyme C3 was shown to inhibit cell motility (Stasia et al. 1991; Takaishi et al. 1994; Santos et al. 1997), cell invasion (Verschueren et al. 1997) and cytokinesis (Kishi et al. 1993; Takaishi et al. 1995; Drechsel et al. 1996).

II. Effects of C3 on Cell–Cell Contacts

Rho proteins participate in the regulation of cell–cell contacts and are important for the barrier functions of epithelial and endothelial cells. In keratinocytes, E-cadherin-dependent adhesions are formed when cells are transferred from a low- to a high-calcium medium. Microinjection of C3 prior to high-calcium incubation selectively removes cadherin complexes from junctions. A similar effect is observed after microinjection of dominant negative N17Rac, indicating that Rho and Rac are necessary for establishment of cad-

herin junctions. In contrast, desmosomes, which contain different members of the cadherin family (desmocollins) and interact with intermediate filaments but not with microfilaments, are not affected by C3 (Braga et al. 1997). C3 also has major effects on tight junctions. In the human intestinal cell line T84, treatment of cells with the C3 toxin chimera DC3B caused destruction of tight-junction function concomitantly with loss of the perijunctional actin ring, redistribution of the tight-junction protein ZO1 and a decrease in trans-epithelial resistance, whereas E-cadherins were not affected in these cells (Nusrat et al. 1995). However, several laboratories showed that the increase in endothelial permeability induced by histamine and thrombin is inhibited by C3 (5 μg/ml, 24 h), indicating the involvement of Rho in this process, most likely by regulating the myosin light-chain phosphorylation and the contraction of the actin–myosin system in a manner very similar to the effects of Rho described above for the contraction of neuronal cells (Essler et al. 1998; Majumdar et al. 1998; Van Nieuw Amerongen et al. 1998).

III. Effects of C3 on Endocytosis and Phagocytosis

C3 and Rho proteins were shown to affect endocytosis. In *Xenopus* oocytes, C3 induced the blocking of constitutive endocytosis of surface sodium pumps. Similarly, C3 or microinjected ADP–ribosylated RhoA inhibited phorbol-ester-induced increase in fluid-phase endocytosis in oocytes, indicating that RhoA is an essential component of a presumably clathrin-independent endocytotic pathway (Schmalzing et al. 1995) and stimulates endocytosis. The effects of C3 were different in studies on clathrin-mediated endocytosis of transferrin and EGF receptors. In permeabilized A431 cells, C3 increased endocytosis (Lamaze et al. 1996). Accordingly, active Rho (also Rac) blocked this type of endocytosis.

Recently, it was shown that C3 blocks the phagocytosis induced by activation of the CR3 integrin receptor (Caron and Hall 1998), indicating the essential role of Rho in this process. In contrast, C3 showed no effect on FcγR-mediated phagocytosis in the same professional phagocytic cells, indicating two distinct mechanisms of phagocytosis: one blocked by C3 and one largely independent of the C3 substrate (Caron and Hall 1998).

IV. Effects of C3 on Cell Signalling not Directly Involving the Actin Cytoskeleton

1. Phospholipase D and PIP5 Kinase

During recent years, it has been realised that Rho proteins are not only involved in the control of the cell architecture and in the dynamic regulation of the actin cytoskeleton (including motility) but also in a large array of signal-transduction processes not directly involved in regulation of the microfilament system. It was shown that PIP5 kinase is an effector of Rho, and C3-induced ADP–ribosylation blocks the activation of the kinase by the GTPase (Chong

et al. 1994). Although the substrate of PIP5 kinase, phosphatidylinositol-4,5-bisphosphate, plays an important role as a substrate in signalling via phospholipase C, regulation of PIP5 kinase by Rho might also connect receptor signalling with the cytoskeleton, because phospholipids regulate the interaction of actin with actin-binding proteins (Hartwig et al. 1995).

Several reports have shown that RhoA regulates phospholipase D (PLD; Siddiqi et al. 1995; Hammond et al. 1997). In HEK cells, C3 and *C. difficile* toxin B, which inactivates Rho GTPases by glucosylation, block PLD stimulation via muscarinic receptors (Schmidt et al. 1996). C3 inhibited the activation of PLD in a rat-brain preparation (Kuribara et al. 1995) and, in MDCK D-1 cells, activity of nuclear PLD was inhibited by C3 (Balboa and Insel 1995). Surprisingly, in a recent report, ADP–ribosylation of Rho had no effect on PLD stimulation by Rho (Smith et al. 1988). This finding is in contrast to the effects of C3 on most other effectors of Rho. Interestingly, recent studies indicate the existence of several phospholipase isoforms, which are most likely differently regulated by Rho GTPases.

2. Signalling to the Nucleus and Gene Transcription

Various Rho GTPases are implicated in the control of gene transcription, cell-cycle progression and cell transformation involving different pathways. C3 was used in several studies to identify the participation of RhoA in transcriptional activation. It was shown that C3 inhibits activation of the transcription factor SRF (serum-response factor) by serum and LPA, which might be the essential factor in serum (Hill et al. 1995; Alberts et al. 1998). Moreover, C3 was successfully employed in delineation of the role of Rho in the signalling (via heterotrimeric G proteins) of various heptahelical receptors to the nucleus C3 inhibited the activation of SRF by α_1-adrenergic-receptor-signalling via $G\alpha_q$ in cardiomyocytes (Sah et al. 1996), by muscarinic m1 receptors via $G\alpha_{12}$ (Fromm et al. 1997) or by thrombin, LPA, thromboxane and endothelin receptors via $G\alpha_{12/13}$ (Mao et al. 1998a, 1998b). Interestingly, $G\alpha_{12}$- and $G\alpha_{13}$-induced stress-fibre formation and focal adhesion assembly is also blocked by C3, emphasising the role of Rho in these effects (Buhl et al. 1995). Microinjection of C3 inhibited cell-cycle progression (Yamamoto et al. 1993) and blocked serum-induced DNA synthesis in Swiss 3T3 fibroblasts (Olson et al. 1995). Recently, C3 was shown to activate the stress-signalling pathways, c-Jun N-terminal kinase and p38 to potentiate c-Jun expression and phosphorylation, but to inhibit the transcriptional activity of activator protein 1 (AP-1). These findings suggest that C3 is a cellular stress which antagonises activation of AP-1 (Beltman et al. 1999).

H. Concluding Remarks

Although much is known about the cellular functions of Rho GTPases, the roles of C3-like transferases in pathogenicity are not at all understood. An

action of C3 exoenzymes on the immune system of the eukaryotic target organism is most likely. Some of these effects have already been mentioned. Inhibition of immune cell functions, including cytotoxicity of lymphocytes (Lang et al. 1992), adhesion (Nemoto et al. 1996), migration and invasion of lymphocytes (Verschueren et al. 1997; Stam et al. 1998) and leukocytes (Laudanna et al. 1996, 1997) by C3, have been demonstrated. Rho GTPases have been proven to be important components of signal pathways used by antigen receptors, co-stimulatory, cytokine, chemotaxin receptors that regulate the immune response (Price et al. 1995; Laudanna et al. 1996; Prepens et al. 1996; Henning and Cantrell 1998; Reif and Cantrell 1998; Wojciak-Stothard et al. 1998). Moreover, Rho proteins participate in the barrier functions of epithelial cells (Nusrat et al. 1995; Vouret-Craviari et al. 1998) and in wound healing (Santos et al. 1997). However, considering the poor cell accessibility of C3 exoenzymes, an important question remains: how do these specific and potent agents get to their site of action? Thus, further studies are necessary to elucidate the role of C3 exoenzymes in pathogenesis.

Rho proteins are not substrates for C3-like transferases only. The family of large clostridial cytotoxins, including *C. difficile* toxins A and B, inactivates Rho proteins by mono-glucosylation (Aktories and Just 1995; Just et al. 1995b, 1995c). These toxins modify all members of the Rho family, including Rac and Cdc42. Moreover, Rho GTPases are the eukaryotic targets for the cytotoxic necrotising factors (CNF1 and CNF2) from *E. coli* and for the dermonecrotic toxin from *Bordetella* species. The latter toxins induce the deamidation of Rho GTPases at Gln63 of Rho (Gln61 of Rac and Cdc42), there by inhibiting the intrinsic and GAP-stimulated GTP hydrolysis and, hence, persistently activating the GTPases (Flatau et al. 1997; Horiguchi et al. 1997; Schmidt et al. 1997). Moreover, recent studies indicate that the list of bacterial toxins/exoenzymes that act on Rho GTPases is even longer. For example, exoenzyme S from *P. aeruginosa*, which is introduced into the eukaryotic cell by a type-III secretion system, appears to alter the actin cytoskeleton by a mechanism suggested to involve Rho (Frithz-Lindsten et al. 1997; Pederson et al. 1998). Another example is the YopE protein from *Yersinia*. This protein is suggested to attack the actin cytoskeleton via Rho GTPases to prevent phagocytosis by macrophages (Mecsas et al. 1998). Finally, SopE, a protein from *Salmonella*, directly activates Rho proteins (Rac, Cdc42) to induce cell-membrane ruffling (Hardt et al. 1998). Thus, Rho GTPases appear to be preferred targets for bacterial toxins and/or exoenzymes.

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CHAPTER 11

Pseudomonas aeruginosa Exoenzyme S, a Bifunctional Cytotoxin Secreted by a Type-III Pathway

J.T. Barbieri and D.W. Frank

A. Introduction

Cystic-fibrosis patients, burn victims, and immunocompromised individuals are particularly susceptible to infection by *Pseudomonas aeruginosa*, a Gram-negative opportunistic pathogen. (Bodey et al. 1983; Roilides et al. 1992; Mendelson et al. 1994). Although *P. aeruginosa* can be controlled with antibiotics, the prevalence of multi-drug-resistant strains has limited the number of antimicrobial agents that are of clinical use (Finland 1972). *P. aeruginosa* possesses an array of virulence factors that contribute to its pathogenesis, including a number of proteases, phopholipases, a neuraminidase, capsular materials, and adhesins (Goranson and Frank 1996; Tummler et al. 1997). In addition to components that aid in adhesin, tissue invasion, and replication within the host, *P. aeruginosa* also encodes three secreted enzymes that possess adenosine diphosphate (ADP)–ribosyltransferase activity: exotoxin A (PAETA), exoenzyme S (ExoS), and exoenzyme T (ExoT).

PAETA is the most toxic exotoxin *P. aeruginosa* produces. The gene encoding PAETA is present in over 95% of clinical isolates. PAETA was identified by Iglewski and coworkers as an ADP–ribosyltransferase that targeted eukaryotic elongation factor 2 (EF-2; Iglewski and Kabat 1975). ADP–ribosylated-EF-2 is unable to perform its translocase function, which results in an inhibition of protein synthesis, and cell death (Iglewski and Kabat 1975; Vasil et al. 1977; Lory and Collier 1980). PAETA is secreted as an inactive proenzyme (Lory and Collier 1980), which is activated by proteolytic cleavage and disulfide-bond reduction, yielding a catalytic carboxyl-terminal peptide. Although PAETA possesses limited primary amino acid homology with other members of the family of bacterial ADP–ribosylating exotoxins (bAREs), the three-dimensional structure of the catalytic domain of PAETA can be superimposed on other bAREs (Sixma et al. 1993). In addition, PAETA contains an active-site glutamic acid (Glu553), which is a signature residue for these exotoxins (Carroll et al. 1980; Ogata et al. 1992). The detailed molecular properties of PAETA are reviewed in a separate chapter.

B. Initial Biochemical Characterization of ExoS

ExoS was identified by Iglewski and coworkers as an ADP–ribosyltransferase of *P. aeruginosa* that possessed unique biochemical and enzymatic activities relative to PAETA (Iglewski et al. 1978). ExoS was purified from the culture supernatant of *P. aeruginosa* as an aggregate composed principally of two proteins with apparent molecular masses of 53 kDa and 49 kDa (Nicas and Iglewski 1984; Coburn 1992; Kulich et al. 1994). The 49-kDa form of ExoS possessed ADP–ribosyltransferase activity and was termed the enzymatically active form of ExoS, while the 53-kDa protein possessed little intrinsic ADP–ribosyltransferase activity. This observation led to the hypothesis that *P. aeruginosa* produced ExoS as a 53-kDa, non-catalytic precursor, which was proteolytically processed to an enzymatically active 49-kDa form. Since the amino-terminal sequences of the 53-kDa and 49-kDa forms of ExoS were essentially identical, post-translational processing was hypothesized to occur at the carboxyl terminus of the 53-kDa form to produce the 49-kDa active form of ExoS. Although this hypothesis was consistent with the known properties of exotoxins with ADP–ribosyltransferase activity, genetic analyses revealed that the two forms of ExoS were encoded by separate loci.

C. Genetic Analysis of the Structural Genes Encoding ExoS

To clone the structural gene encoding ExoS, the enzymatically active 49-kDa form was purified, and segments of the amino acid sequence were generated from amino-terminal and internal tryptic peptides. Degenerate oligonucleotides were designed to amplify the DNA encoding short segments of the structural gene. The gene encoding ExoS, *exoS*, was cloned from a bank of *P. aeruginosa* chromosomal DNA using the amplified DNA fragments as probes (Kulich et al. 1994). Expression analyses in *E. coli* and *P. aeruginosa* demonstrated that recombinant ExoS was expressed as a 49-kDa, enzymatically active protein (Kulich et al. 1995). This observation supported the hypothesis that the 49- and 53-kDa forms were encoded by separate structural genes.

Two experiments demonstrated that the 49-kDa and 53-kDa forms of ExoS were encoded by separate genes. In one study, *exoS* was specifically deleted from the chromosome of *P. aeruginosa* by allelic replacement, and the production of ExoS was measured by Western-blot analysis. The deletion strain, 388Δ*exoS*::Tc, failed to synthesize the 49-kDa protein but still produced the 53-kDa form of ExoS. Second, using *exoS* coding sequences as probes, Southern-blot analysis of chromosomal DNA from 388Δ*exoS*:Tc indicated that a second gene related to *exoS* was encoded on the chromosome. This gene was subsequently cloned and produced a recombinant protein that co-migrated with the 53-kDa form of ExoS, cross-reacted with antibodies specific for ExoS,

and expressed a low but detectable amount of ADP–ribosyltransferase activity for target proteins similar to those acted on by ExoS. Initially, this related gene was termed *exoS53* (YAHR et al. 1996a). The nomenclature was subsequently changed to *exoT* to reflect the fact that there are several extracellular proteins produced by *P. aeruginosa* that are coordinately regulated with the production of ExoS. For historical purposes, the term exoenzyme S is used to refer to both ExoS and ExoT as extracellular enzymes that form an aggregate, since much of the early biochemical work characterized this aggregate.

Relative to other bAREs, ExoS and ExoT possess several unique properties, including the ability to ADP–ribosylate numerous target proteins (COBURN et al. 1989b; COBURN and GILL 1991), the requirement for a eukaryotic accessory protein termed FAS (factor activating ExoS; COBURN et al. 1991) to express ADP–ribosyltransferase activity, and the observation that purified ExoS and ExoT were not cytotoxic to cultured cells. Recent studies have provided insight into the mechanisms responsible for these unique properties of ExoS.

D. ExoS Requires FAS to Express ADP–Ribosyltransferase Activity

COBURN and GILL reported that ExoS possessed an absolute requirement for a eukaryotic protein to express ADP–ribosyltransferase activity, which was termed FAS (COBURN et al. 1991). FAS was cloned from a eukaryotic complementary DNA library and was identified as a member of the 14-3-3 protein family (FU et al. 1993). 14-3-3 proteins have been shown to be ubiquitous within the eukaryotic kingdom, with homologs identified in species from yeast to vertebrates (AITKEN 1995). 14-3-3 proteins contribute to host cell physiology via the regulation of numerous signal-transduction pathways (FU et al. 1993). Recent studies indicate that ExoS possesses intrinsic ADP–ribosyltransferase activity (KNIGHT 1998) and that FAS is an allosteric activator that increases both the affinity of ExoS for nicotinamide adenine dinucleotide (NAD) and the catalytic turnover rate. The activation of ExoS by FAS appears to be kinetically similar to the activation of cholera toxin by ADP–ribosylation factor (BOBAK et al. 1990).

E. Molecular Properties of ExoS

The elucidation of the genetic and biochemical relationships between the 49-kDa and 53-kDa forms of ExoS led to further molecular analyses that established functional domains (Fig. 1). tFASTA analysis showed that ExoS and ExoT shared little overall primary amino acid homology with other members of the family of bAREs. This is a common theme among members of this family of exotoxins. For example, *Escherichia coli* heat-labile enterotoxin (LT)

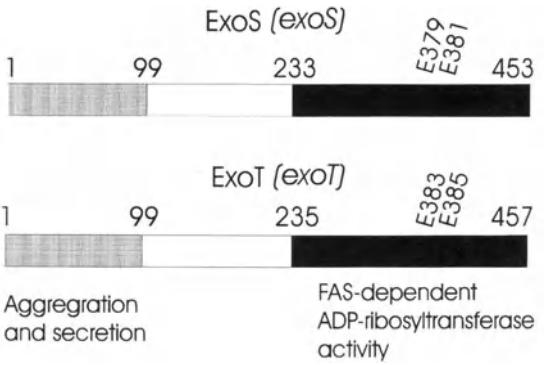

Fig. 1. Molecular organization of exoenzyme S (*ExoS*) and exoenzyme T (*ExoT*). *Pseudomonas aeruginosa* exoenzyme S is composed of two proteins encoded on separate genes. ExoS has 453 amino acids and possesses 76% primary amino acid homology with ExoT, which is composed of 457 amino acids. Both ExoS and ExoT are biglutamic-acid transferases (active-site glutamic acids are noted). The amino terminus is required for the secretion and expression of the aggregation phenotype of the two proteins, while the carboxyl terminus encodes the FAS-dependent adenosine diphosphate–ribosyltransferase domain

and PAETA share common three-dimensional structures within their active site despite the fact that the two proteins possess only three homologous residues among the 43 residues that comprise their active sites (Sixma et al. 1993). One local region of primary amino acid homology, which included Ser343–Thr344–Ser345 of ExoS, was identified between ExoS and several other bAREs. This Ser–Thr–Ser sequence is a conserved component of the active sites of several bAREs. This alignment provided a reference for the subsequent identification of Glu381 as an active-site glutamic acid of ExoS (Liu et al. 1996).

Although ExoS and ExoT possess 76% primary amino acid homology (Yahr et al. 1996a), ExoT catalyzes the ADP–ribosylation of eukaryotic proteins at only 0.2% of the specific activity of ExoS (Yahr et al. 1996a). A kinetic study showed that ExoT possessed a similar binding affinity for NAD and only a fourfold increase in the K_m for target proteins. Thus, it appears that a slower turnover rate is responsible for the low specific activity of ExoT in the expression of ADP–ribosyltransferase activity. ExoT also catalyzed the NAD-glycohydrolase reaction at a much slower specific activity than ExoS, consistent with the idea that ExoT possesses a catalytic defect and not low affinity for target protein. Analysis of chimeric proteins composed of reciprocal halves of the catalytic domains of ExoS and ExoT localized the catalytic defect to the residues between 235 and 349 within ExoT (Liu et al. 1997). Figure 1 outlines the molecular organization of ExoS and ExoT.

F. Secretion of ExoS via a Type-III Secretion Pathway

Once the structural gene for ExoS was cloned and recombinant ExoS was analyzed, it became apparent that purified ExoS had no biological effects in tissue culture or when injected into animals. The simplest explanation for these results, which is consistent with the known structural properties of exotoxins, was that the binding subunit or domain of ExoS was not present in the recombinant materials. Toxicity, however, was not detected with crude extracellular material from *P. aeruginosa* containing ExoS, suggesting that the intoxication mechanism may differ from traditional A:B models. One model that fit the experimental observations was that ExoS might need the bacterium to deliver the molecule. A specialized intoxication mechanism mediated by a bacterial delivery system has been termed type-III secretion. The genes encoding type-III secretion systems have been found in both plant and animal Gram-negative pathogens (Hueck 1998) and contain homologs to genes involved in the assembly, regulation, and secretion of flagellar proteins.

Data to support the hypothesis that ExoS was delivered into susceptible host cells through a type-III pathway was shown in domain-mapping studies. First, ExoS was not processed during secretion, which eliminated a role for the general secretory pathway (type-II secretion) in ExoS secretion. Type-I secreted proteins generally possess a glycine-rich domain within their carboxyl terminus. Since ExoS did not possess a glycine-rich domain, and carboxyl-terminal deletion proteins of ExoS were secretion competent, it appeared that ExoS was not secreted via the type-I secretion pathway. Type-III secreted proteins utilize amino-terminal amino acids or possibly a mRNA recognition sequence for secretion. Consistent with the utilization of a type-III secretion pathway was the observation that amino-terminal deletion proteins of ExoS were not secreted but were retained within the cytoplasm of *P. aeruginosa* (Yahr et al. 1996b; Frithz-Lindsten et al. 1997).

The genes encoding the *P. aeruginosa* type-III secretion system were mapped and sequenced based on their coordinate regulation with ExoS expression (Yahr et al. 1997). The *P. aeruginosa* type-III system is encoded within five operons that are located in the 55-min region of the chromosome. The proteins encoded by the *P. aeruginosa* type-III system possess striking homology to the type-III secretion/translocation system of *Yersinia*. In general, gene order and operon content is highly conserved. Functional conservation between the two type-III systems was demonstrated by the delivery of recombinant ExoS (rExoS) into eukaryotic cells by the *Yersinia* system (Frithz-Lindsten et al. 1997) and the functional complementation of *Yersinia* translocation mutants with *P. aeruginosa* gene products (Frithz-Lindsten et al. 1998).

G. Regulation of *exoS* Regulon Expression

In bacteriological medium, ExoS production is induced by growth of *P. aeruginosa* in the presence of chelators, including ethylene diamine tetraacetic acid, nitrilotriacetic acid, or ethylene glycol tetraacetic acid. Growth in the presence of chelators reduces calcium in the medium, a signal which appears to mimic the interaction with eukaryotic cell surfaces. ExoS production is also induced after growth in the presence of eukaryotic cells or growth in the presence of serum (Vallis 1998). Induction was shown to occur at the level of transcription of the ExoS trans-regulatory locus, which encodes ExsA, a DNA-binding protein that is part of the AraC family of transcriptional activators. ExsA binds to a specific sequence (TxAAAAxA, where x represents any nucleotide) present in the promoter regions of all the operons that constitute the ExoS regulon (Hovey and Frank 1995). These operons include structural, regulatory, translocation, and effector proteins of the *P. aeruginosa* type-III pathway.

H. The Carboxyl Terminus of ExoS Comprises the ADP–Ribosyltransferase Domain

I. Functional Mapping of ExoS

Purified rExoS that was expressed in *E. coli* catalyzed the FAS-dependent ADP–ribosyltransferase reaction at a specific activity that was equivalent to ExoS purified from *P. aeruginosa* (Kulich et al. 1995). This showed that ExoS was necessary and sufficient to express ADP–ribosyltransferase activity and that other *Pseudomonas* proteins did not contribute to the reaction. Characterization of deletion peptides identified the requirement of the amino terminus for efficient secretion of ExoS into the culture medium by *P. aeruginosa* (Knight et al. 1995). In addition, deletion of the amino-terminal 99 amino acids eliminated the aggregation properties of ExoS. One amino-terminal deletion peptide that comprised the 222 carboxyl-terminal amino acids of ExoS (termed ΔN222) catalyzed FAS-dependent ADP–ribosyltransferase reaction at a specific activity that was comparable to that of full-length ExoS. Together, these results indicated that the amino terminus did not contribute to the expression of catalytic activity. Deletion peptides shorter than ΔN222 were not stable or possessed low rates of ADP–ribosyltransferase activity, indicating that ΔN222 represented the catalytic domain of ExoS. ΔN222 is expressed as a stable, soluble peptide in *E. coli* and has been used to characterize the catalytic properties of ExoS.

II. ExoS is a Biglutamic-Acid Transferase

Glu381 was identified as a potential active-site residue of ExoS by alanine-scanning mutagenesis of selected glutamic acids, which were located on the carboxyl-terminal side of the conserved Ser343–Thr344–Ser345 sequence

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                          •   •
LT          108 - P Y E Q E V S A L G
CT          108 - P D E Q E V S A L G
Iota        417 - A G E Y E V L L N H
ExoS        377 - K N E K E I L Y N K
ExoT        381 - G D E Q E I L Y D K

CHAT1       220 - P S E D E V L I P G
HMAT        236 - P G E E E V L I P G
RT6.1       205 - P D Q E E V L I P G
RT6.2       205 - P D Q E E V L I P G
MRT6H       205 - T H E E E V L I P G
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Fig. 2. Alignment of the active-site glutamic acids of several prokaryotic and eukaryotic adenosine diphosphate (ADP)–ribosyltransferases. *Upper alignment*: prokaryotic enzymes. Heat-labile enterotoxin of *Escherichia coli* (*LT*; AC-P43530), cholera toxin (*CT*; AC-P01555), iota toxin of *Clostridium perfringens* (*Iota*; AC-AF037328), *Pseudomonas aeruginosa* exoenzyme S (*ExoS*; AC-L27629) and *P. aeruginosa* exoenzyme T (*ExoT*; AC-L46800). *Lower alignment*: eukaryotic enzymes. Chicken bone-marrow cell ADP–ribosyltransferase 1 (*CHAT1*; AC-D31864), human skeletal-muscle ADP–ribosyltransferase (*HMAT*; AC-P52961), rat T-cell antigen (*RT6.1*; AC-P17982) and (*RT6.2*; AC-P20974) and mouse homologue of RT6 (*MRT6H*: AC-P17981). •Denotes active-site glutamic acids

(Liu et al. 1996). The primary amino acid sequence immediately surrounding Glu381 aligned with several other ADP–ribosyltransferases, including the active-site glutamic acids of CT, LT, iota toxin of *Clostridium perfringens*, and several eukaryotic ADP–ribosyltransferases (Fig. 2). In LT, both Glu110 and Glu112 contribute to the ADP–ribosylation reaction (Lobet et al. 1991) and are located at the entrance to the NAD-binding domain (Sixma et al. 1993; Pizza et al. 1994). ADP–ribosyltransferases like LT have been termed biglutamic-acid transferases, with two catalytic glutamic acids organized in an EXE motif, where E represents the glutamic acids and X represents any amino acid. In contrast, pertussis toxin, diphtheria toxin (DT), and PAETA appear to be mono-glutamic acid transferases, since only a single glutamic acid is required for expression of ADP–ribosyltransferase activity (Pizza et al. 1988; Douglas and Collier 1990; Wilson et al. 1990).

We have recently observed that both Glu379 and Glu381 are required for the efficient ADP–ribosyltransferase activity of ExoS, which identifies ExoS as a biglutamic-acid transferase (Radke 1998). Additional studies showed that, while Glu381 was required for expression of both NAD-glycohydrolase activity and ADP–ribosyltransferase activity, Glu379 is required for expression of only ADP–ribosyltransferase activity. Thus, it appears that the two glutamic acids play unique roles in the catalytic process. Glu381 contributes to an early step in the catalytic reaction mechanism, while Glu379 contributes to a step later in the transferase reaction, possibly the transfer of ADP–ribose to an

arginine residue within the target protein. Thus, Glu381 appears to be the catalytic glutamic acid residue in the transferase reaction. The determination that the Glu381Asp mutant was deficient in both ADP–ribosyltransferase and NAD-glycohydrolase activities while the Glu379Asp mutant was only deficient in ADP–ribosyltransferase activity also allowed a direct determination of the roles of ADP–ribosyltransferase and NAD-glycohydrolase activities in the modulation of host cell physiology. Intracellular expression of neither ΔN222-Glu381Asp nor ΔN222-Glu379Asp caused measurable biologic effects in Chinese hamster ovary (CHO) cells. These data support the hypothesis that expression of ADP–ribosyltransferase activity, but not NAD-glycohydrolase activity, is responsible for the observed cytotoxicity of the ADP–ribosyltransferase domain of ExoS (discussed below).

Enzymes that catalyze the ADP–ribosyltransferase reaction have been proposed to possess a common motif that is comprised of three non-contiguous regions within the primary amino acid sequence (Takada et al. 1995). Alignment of each protein from the amino terminus to the carboxyl terminus allows the mapping of common amino acid residues within regions of the molecules that may contribute to catalysis. The three non-contiguous regions include region I, which contains an essential histidine or arginine residue, region II, which contains aromatic and/or hydrophobic amino acids and includes the Ser–Thr–Ser motif, and region III, which contains the conserved glutamic acid, along with other acidic residues. Our current understanding of the functional properties of ExoS has identified two of the three non-contiguous regions within the common motif of ADP–ribosyltransferases, including region II, which contains Ser343–Thr344–Ser345, and region III, which contains Glu379 and Glu381.

III. ExoS can ADP–Ribosylate Numerous Proteins

Most bAREs ADP–ribosylate specific target proteins. For example, DT and PAETA ADP–ribosylate only EF-2 (Krueger and Barbieri 1995). In contrast, after the addition of FAS, ExoS is capable of covalently modifying numerous proteins in extracts from either prokaryotic or eukaryotic cells (Coburn et al. 1989a, 1989b). The preferred eukaryotic target proteins of ExoS reside within both intracellular and extracellular compartments. Intracellular proteins that are ADP–ribosylated by ExoS include several members of the Ras superfamily of small-molecular-weight guanosine triphosphate (GTP)-binding proteins, and vimentin (Coburn et al. 1989a, 1989b). Recently, ExoS was reported to ADP–ribosylate Ras in vivo (McGuffie et al. 1998) and interfere with a Ras-mediated signal-transduction pathway in PC-12 cells (Ganesan et al. 1998b). Extracellular target proteins ADP–ribosylated by ExoS include serum immunoglobulin G and apolipoprotein A1 (Knight and Barbieri 1997). Although the physiological significance of the ADP–ribosylation of extracellular proteins remains to be determined, ExoS-mediated ADP–ribosyltransferase activity has been identified in extracellular compartments,

such as blood (plasma; NICAS and IGLEWSKI 1984) and pleural fluid (KUDOH et al. 1994) during infection of animal models by *P. aeruginosa* and, more recently, in the tissue-culture medium of eukaryotic cells co-cultured with *P. aeruginosa* (VALLIS 1998).

IV. ExoS ADP–Ribosylates Ras at Multiple Arginine Residues

Ras is a central component of eukaryotic signal transduction, which makes its ADP–ribosylation by ExoS of potential physiological significance. Other bacterial exotoxins post-translationally modify Ras and other members of the Ras superfamily, resulting in the modulation of eukaryotic signal transduction (JUST et al. 1996). ExoS ADP–ribosylated Ras in vitro at a stoichiometry of two molecules of ADP–ribose per molecule of Ras, which suggested that ExoS could ADP–ribosylate Ras at more than one arginine residue (GANESAN et al. 1998a, 1998b). Utilizing in vitro transcribed/translated Ras, Arg41 was identified as the preferred site of ADP–ribosylation. While ExoS ADP–ribosylated Ras at a second site, it appeared that this second ADP–ribosylation did not occur at a specific arginine residue (GANESAN et al. 1998a). Subsequent studies showed that ExoS double ADP–ribosylates Ras at Arg41 and Arg128 (GANESAN 1998a; Fig. 3). Analysis of the double mutant, Ras

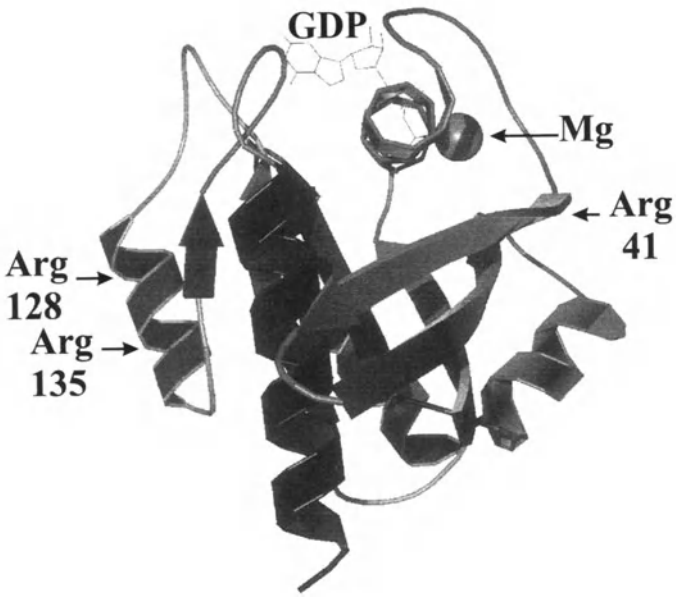

Fig. 3. Exoenzyme S (ExoS) adenosine diphosphate (ADP)–ribosylates Ras at multiple sites. This ribbon diagram shows the two arginines of Ras that are ADP–ribosylated by ExoS, Arg41 and Arg128. Arg41 is located on a β-strand within the switch-1 region, and Arg128 is located within an α-helix. Arg135 is located within the same α-helix as Arg128 and is an alternate site of ADP–ribosylation by ExoS

```
          32                  50      123          137
Rap1a     YDPTIEDSYR.KQVEVDCQQ.....RVVGKEQGQNLARQW
Rap1b     YDPTIEDSYR.KQVEVDAQQ.....RVVGK EQGQNLARQW
Rap2      YDPTIEDFYR.KEIEVDSSP.....REVSS SEGRALAEEW
Ras       YDPTIEDSYR.KQVVIDGET.....RTVES ROAODKARSY
Ral       YEPTKADSYR.KKVVLDGEE.....RQVSV EEAKNRAEQW
Rab3b     FVSTVGIDFKVKTVYRHEKR.....RVVPT EKGQLLAEQ
Rab4a     SNHTIGVEFGSKIINVGGKY.....REVTF LEASRFAQE
RhoA      YVPTVFENY.VADIEVDGKQ.....EPVKP EEGRDMANRI
```

Fig. 4. Alignment of members of the Ras superfamily with respect to Arg41 and Arg128 of Ras. Using the Pileup program (Genetics Computer Group), regions of members of the family of small-molecular-weight guanosine triphosphate (GTP)-binding proteins corresponding to sites of adenosine diphosphate (ADP)–ribosylation of Ras were aligned. Individual members of the family of small-molecular-weight GTP-binding proteins are indicated to the left of the alignment. The sites of ADP–ribosylation of Ras are shown in *bold underline*, and potential sites of ADP–ribosylation within other members of this family are shown in *bold*

Arg41Lys,Arg128Lys, revealed an alternative site for ADP–ribosylation by ExoS as Arg135. Since ExoS did not ADP–ribosylate Arg135 in wild-type Ras, it appears that the ADP–ribosylation at Arg128 sterically blocked the ADP–ribosylation of Arg135. This indicates that ExoS can bind Ras in several orientations to facilitate ADP–ribosylation along the α-helix containing Arg128 and Arg135. ExoS ADP–ribosylates Ras at arginine residues on two distinct surfaces of Ras, the β-sheet containing Arg41 and the α-helix containing Arg128 and Arg135 (Fig. 3). These data are consistent with the presence of two independent binding events to achieve double ADP–ribosylation.

The plasticity of the ExoS–Ras interactions may explain the observed ability of ExoS to ADP–ribosylate numerous small-molecular-weight GTP-binding proteins in vitro (Coburn et al. 1989b). Alignment of members of the Ras superfamily showed that a limited subset of the family (Ras, Ral, and Rap) contained the Arg41 homologue. In contrast, alignment of the α-helix, which contains Arg128, the second site ADP–ribosylated by ExoS, identified several members of the Ras superfamily with potential sites for ADP–ribosylation. However, no distinct recognition motif is apparent upon examination of the primary amino acid sequences of members of the Ras superfamily (Fig. 4). The potentials of ADP–ribosylating Ras superfamily members at their Arg41 or Arg128 homologues may have functional implications.

I. ExoS is a Bifunctional Cytotoxin

The ability to independently express the amino- and carboxyl-terminal domains of ExoS has increased the resolution of the functional mapping of

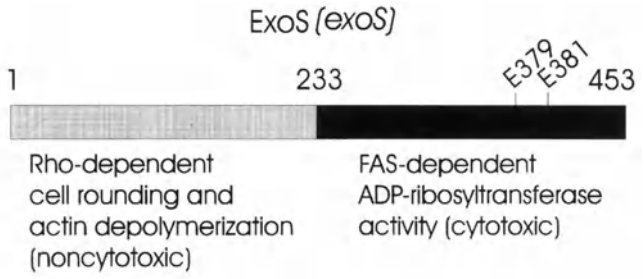

Fig. 5. Functional domains of exoenzyme S (*ExoS*). The schematic demonstrates the bifunctional properties of ExoS. The amino-terminal 234 amino acids elicit a Rho-dependent depolymerization of actin, while the carboxyl-terminal 222 amino acids comprise the FAS-dependent adenosine diphosphate–ribosyltransferase domain. *E379* and *E381* correspond to the biglutamic acids involved in catalysis

ExoS and has allowed the determination that ExoS is a bifunctional cytotoxin (Fig. 5).

I. Cytotoxic Properties of ExoS

Attempts to intoxicate eukaryotic cells with purified ExoS have been unsuccessful (Coburn 1992; Olson et al. 1997). These earlier observations can be explained by the identification of genes encoding a type-III secretion apparatus within the *exoS* regulon (Yahr et al. 1996b). To determine the role of *P. aeruginosa* adhesion in ExoS-mediated intoxication of cultured cells, a co-culture infection model was developed in Detroit 532 fibroblastic cells (Olson et al. 1997). In this model, *P. aeruginosa* expressing ExoS caused a greater decrease in DNA synthesis and cell viability than strains of *P. aeruginosa* that lacked the gene encoding ExoS. These results supported studies with ExoS expressed by *Yersinia pseudotuberculosis* (Frithz-Lindsten et al. 1997). Using *Yersinia* as a heterologous delivery system, ExoS exerted a cytotoxic effect in HeLa cells. In these experiments, cytotoxicity was defined as the ability to elicit rounding of cells. Delivery of ExoS was also dependent on the presence of a functional YopB protein, which indicated that ExoS was translocated in a manner similar to Yops. While the amount of cytotoxicity correlated to the level of ADP–ribosyltransferase activity, infection of HeLa cells with a *Yersinia* strain that expressed a non-catalytic form of ExoS elicited an intermediate cytotoxic phenotype.

II. The Amino Terminus of ExoS Stimulates Rho-Dependent Depolymerization of Actin

To enhance the resolution of the effect of ExoS on the physiology of eukaryotic cells, a transient cytomegalovirus expression vector was developed to

transfect cultured cells with plasmids encoding specific forms of ExoS. Intracellular expression of the amino-terminal 234 amino acids of ExoS in CHO cells elicited the depolymerization of actin via a Rho-dependent mechanism (Pederson 1998a). Analysis of reporter-protein expression and trypan-blue staining showed that the depolymerization of actin by expression of the amino terminus of ExoS was not cytotoxic to eukaryotic cells.

Although the in vivo role of ExoS in host pathogenesis remains to be fully elucidated, the amino terminus of ExoS shares limited homology with the YopE cytotoxin of *Yersinia* and the amino terminus of SptP of *Salmonella* (Yahr et al. 1996b; Collazo and Galan 1997; Frithz-Lindsten et al. 1997). YopE is a member of a group of effector proteins that are secreted and translocated by *Yersinia* into eukaryotic cells via a type-III-dependent secretion pathway and serve primarily to protect *Yersinia* from the host immune system (Cornelis and Wolf-Watz 1997). YopE inhibits phagocytosis of *Yersinia* by macrophages and was identified as a cytotoxin due to its ability to alter cell morphology and depolymerize actin filaments in HeLa cells (Rosqvist et al. 1990, 1991). Recent studies demonstrating inhibition of bacterial cell entry by YopE with various inhibitors of cell signaling implicated a role for Rho in the action of YopE on eukaryotic cells (Mecsas et al. 1998).

III. The Carboxyl Terminus of ExoS is an ADP–Ribosyltransferase that is Cytotoxic to Cultured Cells

Intracellular expression of the carboxyl-terminal 222 amino acids of ExoS, which includes the ADP–ribosyltransferase domain, was cytotoxic to eukaryotic cells (Pederson 1998b). Intracellular expression of ΔN222 reduced the expression of two independent reporter proteins and decreased trypan-blue exclusion. In contrast, intracellular expression of a non-catalytic form of ΔN222, which expressed reduced ADP–ribosyltransferase activity, elicited little inhibition of reporter-protein expression and little cytotoxicity in transfected cells. These data indicated that expression of the ADP–ribosyltransferase domain of ExoS is cytotoxic to cultured cells.

J. Mechanism for the Inhibition of Ras-Mediated Signal Transduction by ExoS

Recent studies have addressed the mechanism of the inhibition of Ras-mediated signal transduction by ExoS, which was observed in PC-12 cells (Ganesan et al. 1998a). In these experiments, Ras was double-ADP–ribosylated by ExoS, and the physiological properties of ADP–ribose–Ras were compared with those of native Ras. ADP–ribosylation of Arg41 could modulate several steps in Ras-mediated signal transduction, including interactions with Raf or nucleotide exchange. Initial experiments showed that ADP–ribosylated Ras possessed the same binding capacity for Raf as native

Ras, which indicated that ADP–ribosylation did not interfere with Ras–Raf interactions. Although ADP–ribosylated Ras and native Ras possessed similar capacities for intrinsic nucleotide exchange, ADP–ribosylated-Ras possessed a lower rate of guanine-nucleotide-exchange-factor-mediated nucleotide exchange relative to native Ras (GANESAN 1998b). Thus, it appears that ADP–ribosylation at Arg41 interferes with growth-factor-stimulated signal transduction in eukaryotic cells. This mechanism of inhibition of Ras-mediated signal transduction by ExoS is not found in other exotoxins. Future studies will address whether or not this inhibition is solely responsible for the observed effects of expression of ADP–ribosyltransferase activity by ExoS.

K. Functional and Sequence Relationship Between ExoS and the Vertebrate ADP–Ribosyltransferases

One unexpected observation that has been made during the characterization of ExoS is that it shares some functional and molecular properties with the vertebrate ADP–ribosyltransferases. To date, several vertebrate ADP–ribosyltransferases have been identified, including rabbit skeletal-muscle ADP–ribosyltransferase, rat RT6, human ecto-ADP–ribosyltransferase, and chicken ADP–ribosyltransferase types I and II. Properties common to ExoS and the vertebrate ADP–ribosyltransferase include the ability to ADP–ribosylate multiple target proteins and the ability to ADP–ribosylate more than one arginine residue within a target protein. The tFASTA algorithm showed that ExoS could be aligned more extensively with the vertebrate ADP–ribosyltransferases than the bAREs, which extended from region II and included sequences surrounding the Ser–Thr–Ser motif through the catalytic glutamic acids of region III. Figure 6 shows a tFASTA alignment between the

```
SCORES      Init1:    33 Initn:    33 Opt:    81
Smith-Waterman score: 85;    31.9% identity in 116 aa overlap

               280       290       300       310       320
ExoS    YTGIHYADLNRALRQGQELDAGQKLIDQGMSAAFEKSGQAEQVVKTFRGTRGGDAFNA
        | ::||  |:|||          | | |||  :::: ::     |: || |  |::
RT6     YKAFHYY-LTRAL---------QLLSDQGCRSVYRGTN-----VR-FRYTGKGSV---
                130                140             150        160

        330       340       350           360       370       380
ExoS    VEEGKVGHDDGYLSTSLNPGVARS--F--GQGTISTVFGRSGIDVSGISNYKNEKEIL
            : ||   : |:||| :|| |  |  ||||:  :    |  ::  | | :|:|:|
RT6     ----RFGH---FASSSLNRSVATSSPFFNGQGTLFIIKTCLGAHIKHCSYYTHEEEVL
                     170       180       190       200       210
```

Fig. 6. Primary amino acid alignment between the catalytic domain of exoenzyme S (ExoS) and a vertebrate adenosine diphosphate–ribosyltransferase, RT6. Using the tFASTA algorithm, an alignment was observed between region II and region III of ExoS and RT6. This region shows 31.9% homology over 116 amino acids

catalytic portion of ExoS and the vertebrate ADP–ribosyltransferase, RT6. Characterization of vertebrate RT6.1 showed that this protein possessed only NAD-glycohydrolase activity and that conversion of Gln379 to Glu379 resulted in the ability of the RT6.1 to catalyze the ADP–ribosyltransferase reaction, which was consistent with RT6 being a biglutamic-acid transferase (Hara et al. 1996). In contrast, alignment of ExoS with the bAREs showed primary amino acid homology centered within the Ser–Thr–Ser sequence of region II and did not extend to the catalytic glutamic acids of region III. These data suggest that ExoS may have an evolutionary link with the vertebrate ADP–ribosyltransferases.

L. Conclusion

ExoS possesses several properties which are unique among members of the family of bAREs. ExoS is secreted via a type-III secretion pathway, requires a eukaryotic protein for expression of catalytic activity, and can target multiple proteins for ADP–ribosylation. Recent investigations have begun to resolve the molecular properties of ExoS and indicate that ADP–ribosylation interferes directly with Ras-mediated signal transduction. Future experiments will continue to address the significance of the bifunctional properties of ExoS and its role in the pathogenesis of *P. aeruginosa*.

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CHAPTER 12

Structure and Function of Actin–Adenosine-Diphosphate-Ribosylating Toxins

I. OHISHI

A. Introduction

Anaerobic and spore-forming clostridia produce various types of toxins. For almost two decades, the novel molecular structures and enzyme activities of clostridial toxins have been intensively studied. The toxins that are composed of neither covalently nor noncovalently linked proteins and that specifically adenosine-diphosphate (ADP)-ribosylate muscle and/or non-muscle actins are a new category of the bacterial toxin. In this chapter, I shall focus on research on these new bacterial toxins, especially on the molecular structures and functions of the clostridial actin-specific ADP-ribosylating toxins. To date, the actin-specific ADP-ribosylating toxins elaborated by the bacteria other than clostridia have not yet been explored.

B. *Clostridium Botulinum* C_2 Toxin

I. Actin–ADP-Ribosylating Toxin of *C. Botulinum* Types C and D

C. botulinum is known to produce an extremely potent neurotoxin. In the 1920s, two strains of *C. botulinum* type C were isolated. One was isolated from green-fly larvae by BENGTSON in United States, and one was isolated from cattle by SEDDON in Australia (BENGTSON 1922; SEDDON 1922). BENGTSON and SEDDON'S strains were designated as Cα and Cβ, respectively, because anti-toxin sera to Cα and Cβ strains did not completely cross-neutralize (GUNNNISON and MEYER 1929). Thereafter, the antigenically distinct toxins produced by Cα and Cβ strains were designated as C_1 and C_2 toxins; Cα produced C_1 and C_2 in addition to a small amount of type-D toxin, whereas Cβ produced C_2 toxin only (MASON and ROBINSON 1935; JENSEN 1971). However, C_2 toxin produced by either Cα or Cβ strains was considered to be one of the botulinum neurotoxins until the C_2 toxin was purified and characterized in 1980 (OHISHI et al. 1980).

C_2 toxin is produced by some, but not all, *C. botulinum* type C and D strains and by "nontoxigenic" *C. botulinum* type C, which produces no C_1 or D toxin. These nontoxigenic strains were induced by curing prophages with UV light or other suitable agents and were isolated from soil (INOUE and IIDA 1970; EKLUND et al. 1971; NAKAMURA et al. 1978). The purified C_2 toxin con-

sists of two distinct proteins, which are linked by neither covalent nor noncovalent bonds. In 1986, one of the two components of C_2 toxin turned out to be an actin-specific ADP-ribosyltransferase (Aktories et al. 1986; Ohishi and Tuyama 1986). These findings revealed that C_2 toxin is entirely different from botulinum neurotoxin in terms of molecular structure and biological activity.

II. Molecular Structure of Botulinum C_2 Toxin

The evidence that C_2 toxin is composed of two dissimilar, non-linked proteins was obtained by chromatographic separations of the toxin in the culture supernatant of *C. botulinum* type C strain 92-13. Substances with little or only weak toxicity were eluted from chromatography columns, gel filtrations and ion-exchangers (Iwasaki et al. 1980). However, a substance with almost full toxicity was recovered when two different fractions were combined, implying that C_2 toxin consists of two protein components, which individually have extremely low activity and can be resolved into two fractions by chromatography. These two proteins were purified from nontoxigenic *C. botulinum* type C strain 92-13 and were designated components I and II because of the order of their elution from CM-Sephadex columns at pH 6.0. Each of components I and II gave a single band in sodium dodecyl sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) both before and after treatment with reducing agents, indicating that both proteins have no subunit structure. The same molecular structure was proved with the toxins produced by *C. botulinum* type C and D strains (Ohishi 1982; Ohishi and Okada 1986).

The molecular sizes of purified components I and II, as determined by SDS-PAGE, were 55 kDa and 105 kDa, respectively (Ohishi et al. 1980). Recently the genes encoding both components I and II were cloned from nontoxigenic *C. botulinum* type C strain 203U28, which was induced by UV-irradiation of toxigenic type C strain 203 and produces no C_1 or D toxin (Fujii et al. 1996; Kimura et al. 1998). The molecular masses of components I and II, calculated from the deduced amino acid sequence, were 49.40037 kDa and 80.52550 kDa, respectively. A large difference between the molecular weight of component II calculated with SDS-PAGE and that calculated from the cloned gene seems to be due to heterogeneity in the molecular size of component II, which depends on the C_2-toxin-producing strain used (Ohishi and Okada 1986; Ohishi and Hama 1992). The gene for component II of strain 203U28 was homologous to that of *C. botulinum* type D strain 1873, which produced a component II weighing 95 kDa (instead of 105 kDa), according to SDS-PAGE studies. The primary structures of components I and II of C_2 toxin, as deduced from the cloned genes, are compared with the structure of iota toxin in Sect. C.II.

The two components of C_2 toxin produced by different strains of *C. botulinum* types C and D differed in size and in biological activity (Ohishi and Hama 1992). Based on the differences in antigenicity, molecular size and biological activity, the C_2-toxin producers were divided into three groups (Ohishi

and Okada 1986; Ohishi and Hama 1992). Little or no activity of C_2 toxin was detected in culture supernatants of the strains of the third group. Because the two components were detected by agar-gel diffusion and immunoblotting in the culture supernatant, and because the activity was restored when component I of strain 92-13 was added to the culture supernatant, it seems that component I of the third group is produced in an inactive form (Ohishi and Hama 1992).

C_2 toxin is produced as a precursor, and the toxicity is enhanced by treating the toxin with trypsin (Ohishi 1987). It was possible to examine the activity of the toxin simply by combining the trypsinized component with the non-trypsinized one, because C_2 toxin is composed of two non-linked protein components. The activation of C_2 toxin was attained by a mixture of non-trypsinized component I and trypsinized component II, but not by that of trypsinized component I and non-trypsinized component II. Therefore, the activation of C_2 toxin is attributable to the molecular cleavage of component II by trypsin. As determined by SDS-PAGE analysis, the trypsinization of component II was always accompanied by a decrease in molecular size from 101 kDa to 88 kDa (Fig. 1). The trypsinized component II was resolved into two peaks by gel filtration (Ohishi 1987). The molecular sizes of these peaks (as determined by gel filtration) were 365 kDa and 74 kDa, respectively, while that of non-trypsinized component II was 84 kDa. The 365-kDa component II showed hemagglutination and hemolytic activity, whereas that of 74 kDa exhibited only hemagglutination activity; non-trypsinized component II had neither activity. The proteolytic cleavage of component II of C_2 toxin may be an essential step in creating a divalent molecule during the binding of the toxin to the cell membrane.

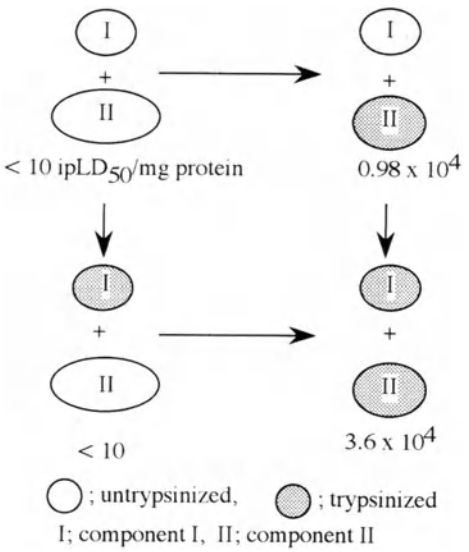

Fig. 1. Lethal activity of botulinum C2 toxin in mice, caused by combining each of trypsinized or non-trypsinized components I and II (*I*, *II*). *Open areas*, untrypsinized; *hatched areas*, trypsinized

III. Molecular Functions of Two Components of C_2 Toxin

Each of the two components of botulinum C_2 toxin, components I and II, has a different function, although the cooperation of the two is essentially requisite for activity in an assay system for whole cells. At present, it is known that component I has actin-specific ADP-ribosyltransferase (the "active component"), whereas component II binds to cell membranes (the "binding component"). These specialized functions of the two components are very similar to the well-known molecular structures of the other bacterial toxins, e.g. cholera, pertussis and diphtheria toxins. In the A–B model of bacterial toxins proposed by Gill, the two components of C_2 toxin correspond to A (active) and B (binding) subunits (GILL 1987). However, C_2 toxin differs from this toxin model in that the two components of the toxin are not linked by covalent or noncovalent bonds (OHISHI et al. 1980). Therefore, the question of how these two non-linked components cooperate in eliciting biological activity arises.

C_2 toxin, a mixture of non-trypsinized component I and trypsinized component II, showed enterotoxic activity when injected into ligated intestinal loops of mice, although neither component alone showed any activity (OHISHI 1983b). In addition, the activity was positive even when the intestinal loop was washed to remove free toxin immediately after injection of the toxin. This suggests that the activity is induced by the binding of the two components to cell surfaces in the intestine. This is supported by the finding that component II (both trypsinized and non-trypsinized) binds to the isolated epithelial cells of mouse intestine, as examined by immunofluorescence staining. The cell-bound component II is highly localized on brush borders of epithelial cells. Component I bound to the cells only when they were exposed to trypsinized component II (but not to the non-trypsinized components). The binding of these components was very rapid and was dependent on their concentrations (OHISHI 1985). Therefore, the two components of C_2 toxin act as dual-functional molecules, with trypsinized component II as the recognizer of target cell membranes and component I as the effector in the cytoplasm. This was further supported by the cytopathogenic effects of the toxin on cultured CMK cells, whose characteristic response to the toxin is rounding (MIYAKE and OHISHI 1987). C_2-toxin component I or II alone or a combination of non-trypsinized components I and II induced little or no response in cells, whereas a mixture of component I and trypsinized component II induced marked response. As determined by immunofluorescence staining, component I bound to the cells only in the presence of trypsinized component II, although both trypsinized and non-trypsinized component II bound to the cells. In vascular permeability assays of C_2 toxin in guinea pigs, the activity was positive only at the site of injection of activated component II when the two components were injected intradermally into separate sites (OHISHI 1983a). Moreover, by injecting one component intradermally and the other intravenously, the intradermal injection site of trypsinized component II (but not the site of component I) showed a positive response. All of these results indicate that the binding of trypsin-activated

component II to the cell membrane induces the binding site for component I, which enters the cells and subsequently induces cellular damage.

Receptor-mediated endocytosis of the two components of C_2 toxin in cultured Y-1 cells was reported by SIMPSON (1989). When the cells were exposed to component II at 37°C and then incubated at 4°C, the cells were no longer accessible to component I. Antagonists for receptor-mediated endocytosis, such as ammonium chloride and methylamine hydrochloride, inhibited the rounding of the cells (SIMPSON 1989). In cultured Vero cells, binding and internalization of the two components of C2 toxin were visualized using fluorescently labeled components I and II (OHISHI and YANAGIMOTO 1992). Component I was located with trypsinized component II on cell membranes during an early period of the incubation. On further incubation, the two components were found in the same cytoplasmic vesicles, indicating that components I and II of C_2 toxin enter the cells through the same route and in the same endosomes. Both non-trypsinized and trypsinized component II of the toxin apparently bind to the same sites of the cell membrane, because the two components are competitive in binding the fluorescently labeled and unlabeled components.

The activated (but not the non-activated) component II of C_2 toxin was able to induce the formation of small ion-selective channels in artificial lipid-bilayer membranes (SCHMID et al. 1994). This suggests that channel formation is one of the steps of the translocation of the toxin through the cell membrane, because several bacterial toxins that are known to enter the cells by receptor-mediated endocytosis also induced pore formation. Recently, the isolation of a C_2-toxin-resistant cell line induced by mutagenesis was reported (FRITZ et al. 1995). The cytoplasmic actin of these mutant cells remained sensitive to ADP-ribosylation by C_2 toxin and other cytopathogenic bacterial toxins, suggesting that only the binding site for component II of C_2 toxin on the cell membrane is impaired in these cells. However, little is known about the mechanisms underlying the binding site and the translocation of C_2 toxin into the cells.

IV. ADP-Ribosylation of Actin by C_2 Toxin

In 1984, SIMPSON reported that component I (which he designated the "light chain") of C_2 toxin possessed the ability to ADP-ribosylate the synthetic substrate homo-poly-L-arginine, indicating that the component I is an enzyme. Component I also ADP-ribosylates protein(s) in the supernatants or pellets from homogenates of several mouse tissues, suggesting that the modified site of the intracellular protein(s) is an arginine residue or something structurally closely related (SIMPSON 1984). However, the target protein modified by component I was not identified. In 1986, the substrate for component I was identified as intracellular actin (AKTORIES et al. 1986a; OHISHI and TUYAMA 1986). Component I of C_2 toxin ADP-ribosylated non-muscle actin (β/γ-actin isoforms) purified from rabbit brain but did not ADP-ribosylate muscle actin (α-actin isoform) from rabbit muscle. Actin monomers (G-actin) – but not

polymers (F-actin) – were modified by component I, implying that the ADP-ribosylation site of actin monomer in the polymerized form is inaccessible to component I, because the site is folded inside a linear double helix of actin polymers (Aktories et al. 1986a). In an in vitro assay, the ADP-ribosylated actin is incapable of polymerization, indicating that the covalent modification of non-muscle actin monomers by component I alters at least one of the functional activities of the actin (Aktories et al. 1986a). Actin is one of the most abundant proteins in eukaryotic cells. It is involved in various cellular motile functions, such as locomotion, cytoplasmic streaming, endocytosis and exocytosis, and plays an important role in the organization of cell architecture and cell-to-cell interaction. These diverse functions of actin are made possible by its ability to polymerize and depolymerize. Therefore, the covalent modification and subsequent inactivation of intracellular actin monomers by C_2 toxin can easily be linked to marked cytopathic effects in cells. Thus, the ADP-ribosylation of cytoplasmic actin by component I of C_2 toxin explains a variety of cellular responses to the toxin.

The changes in the biological activities of cells exposed to C_2 toxin have been investigated in various systems, including whole animals, intact organs and cultured cells. Since this review does not aim to cover all of these areas, they are listed in Table 1, which is useful when determining the intracellular function(s) of actin by using C_2 toxin as a tool (especially when applying the toxin to the outsides of the cells).

1. ADP-Ribosylation of Intracellular Actin of Cultured Cells

The ADP-ribosylation of intracellular actin by C_2 toxin correlates well with the rounding of cultured CMK cells (Ohishi and Tuyama 1986). The rounding of CMK cells depends on the concentration of C_2 toxin after a latent period for 0.5–1 h; again, components I or II alone show very low activity (Miyake and Ohishi 1987). The morphological changes in cultured Y-1 adrenal cells were not accompanied by an increase or decrease in cyclic-AMP or cyclic-guanosine-monophosphate levels (Zepeda et al. 1988). Moreover, no inhibitory effect of C_2 toxin on the incorporation of ^{14}C-leucine, ^{3}H-uridine or ^{3}H-thymidine was observed (Miyake and Ohishi 1987; Zepeda et al. 1988). In fluorescein–phalloidin staining of stress fibers, the microfilament network in intact chick embryo cells was disorganized by C_2 toxin, depending on the incubation period (Reuner et al. 1987). C_2 toxin induced a disruption of cytoskeletal microfilaments and intermediate filaments without affecting microtubules in cultured rat-hepatoma FAO cells (Wiegers et al. 1991). The G-actin pool in intact chicken cells increased, depending on the degree of ADP-ribosylation of intracellular actin (Aktories et al. 1989). All of these results show that ADP-ribosylation of intracellular actin monomers by C_2 toxin causes the depolymerization of F-actin, thereby resulting in the disassembly of the cytoskeletal network and, subsequently, in morphological changes in cultured cells.

Table 1. Biological activities of botulinum C_2 toxin in the whole animals, the organs and the tissue-cultured cells. The activities assayed with purified C_2 toxin are listed chronologically

Biological activity	Whole animals/organs/cells	References
Lethality by intravenous injection	Mice	Ohishi et al. 1980
Vascular permeability by intradermal injection	Guinea pigs	Ohishi et al. 1982
In vitro toxicity	Mice, rats, guinea pigs and chicks	Simpson 1982
Paralysis in neuromuscular transmission	Isolated neuromuscular preparations	
Drop in blood pressure and heart rate	Rat heart	
Gross pathological findings	Mice and rats	
Enterotoxic activity	Mouse intestine	Ohishi 1983b
Histopathological effect	Mouse intestine	Ohishi and Odagiri 1984
Cytopathic activity (rounding activity)	Cultured BHK-21, FL, HeLa, RK13, Vero, CHO-K1, intestine 407 and L929 cell lines	Ohishi et al. 1984
Inhibition of histamine release	Rat mast cells	Böttinger et al. 1987
Disorganization of microfilaments	Chick embryo cells	Reuner et al. 1987
Activation of oxidase	Rat neutrophils	Al-Mohanna et al. 1987
Rounding of the cells	Cultured CMK cells	Miyake and Ohishi 1987
Rounding of the cells	Cultured Y-1 adrenal cells	Zepeda et al. 1988
FMLP-stimulated superoxide-anion production	Human neutrophils	Norgauer et al. 1988
Exocytosis; stimulated noradrenaline release	PC12 cells	Matter et al. 1988
Inhibition in muscle contraction	Longitudinal muscle of guinea pig	Mauss et al. 1989
Disassembly of cytoskeletal filaments	Rat hepatoma FAO and glioma U333 cells	Wiegers et al. 1991
Increase in endothelial permeability	Monolayer of pulmonary-artery endothelial cells	Suttorp et al. 1991
Chemoattractant-evoked PMN activation	Human neutrophils (PMN)	Grimminger et al. 1991
Chemoattractant-evoked cellular signal transduction	Human neutrophils (PMN)	Grimminger et al. 1991
Steroid production	Cultured Y-1 adrenal cells	Considine et al. 1991
Morphological changes	Cultured endothelial cells of porcine artery	Müller et al. 1992
Inhibition of stimulated insulin secretion	Cultured HIT-T15 cells and pancreatic islets	Li et al. 1994
Inhibition of in vitro invasiveness and motility	Murine T-lymphocyte cell line	Verschueren et al. 1995

Table 1. *Continued*

Biological activity	Whole animals/organs/cells	References
Increase in permeability of perfused rabbit lungs	Rabbit lungs	Ermert et al. 1995
Toxin-resistant cell line	CHO-K1 cells	Frits et al. 1995
Microinjection of ADP-ribosylated actins	PtK2 cells	Kiefer et al. 1996
Inhibition in actin synthesis	Hepatoma	Reuner et al. 1996
Microfilament morphology and migratory behavior	Fibroblasts and myofibroblasts	Ronnov-Jessen and Petersen 1996
Ultrastructural changes in endothelium	Perfused rabbit lungs	Ermert et al. 1997
Degranulation and depolymerization of actin filaments	Rat peritoneal mast cells	Wex et al. 1997
Exocytosis and superoxide-anion formation	Human neutrophils	Wenzel-Seifert et al. 1997
FcεRI-mediated serotonin release	RBL cells	Prepens et al. 1998

ADP, adenosine diphosphate; *FMLP*, formyl-methionyl-leucyl-phenylalanine; *PMN*, polymorphonuclear neutrophil; *RBL*, rat basophilic leukemia.

2. ADP-Ribosylation of Purified Actin

ADP-ribosylation of purified human platelet actin by component I of C_2 toxin abolished the formation of typical F-actin, as observed by electron microscopy, although gravel-like structures were formed with ADP-ribosylated actins under polymerization conditions (Aktories et al. 1986b). Component I modified both β- and γ-actins of hog liver, and the ADP-ribosylation site of the actin was located at Arg-177, which is the same as the modification site for both muscle (α-actin) and non-muscle (β/γ-actin) actins by *C. perfringens* type E iota toxin (Vandekerckhove et al. 1987, 1988; Sect. C). Vandekerckhove et al. suggested that the substrate specificity for component I is possibly determined by the amino acid residue preceding the ADP-ribosylated Arg-177. The amino acid residue preceding Arg-177 is methionine in muscle actin and leucine in non-muscle actin (Vandekerckhove et al. 1988). They mentioned another possible reason for the substrate specificity; the topographical structure far from the target residue may influence modification by C_2-toxin component I. This was partly evidenced by ADP-ribosylation of purified *Drosophila* indirect-flight-muscle actin by component I (Just et al. 1993). By cloning the *Act88F* gene, which encodes the indirect-flight-muscle actin, they prepared several mutants: C-terminal mutants (E334K, V229I, E364, G368 and R372H) and L176M, R177Q and Δ0–12. Actins from the R177Q mutant and the N-terminal 0–12 deletion mutant were not ADP-ribosylated by component I of C_2 toxin, whereas the others were modified. The results show that

Arg-177 is the modification site of indirect-flight-muscle actin, and the preceding leucine is not essential for substrate specificity. Moreover, the N-terminal region may be requisite for the modification, whereas some of C-terminal residues (Glu-334, Val-339, Glu-364, Gly-368 and Arg-372) are not crucial for modification by component I of C_2 toxin. The γ-smooth-muscle actin differs from α-smooth-muscle actin only in three amino acid residues at the N-terminal end; γ-smooth-muscle actin also differs from α-skeletal, α-cardiac and α-smooth-muscle actins at an additional amino acid residue at the N-terminal end. Therefore, the substrate specificities of clostridial ADP-ribosyltransferases may be ascribable to differences in the N-terminal structures of the actins.

Many cellular functions are undoubtedly controlled by reversible polymerization/depolymerization of cytoplasmic actins. Actin filaments have polarity, and the rate constants for monomer association or dissociation at both ends are different; the filaments grow faster at the barbed end than at the pointed end. The effect of actin–ADP-ribosylation on the equilibration between polymerization and depolymerization was first demonstrated with rabbit skeletal muscle actins modified by *C. perfringens* iota toxin (Wegners and Aktories 1988; Sect. C). The polymerization of actin monomers was inhibited, depending on the concentration of ADP-ribosylated actin, while the polymerized actin was dissociated in the presence of modified actins. Because polymerization at the pointed ends of actin filaments (the barbed ends of which were capped with gelsolin) was not affected by the presence of ADP-ribosylated actin, the inhibition of actin polymerization in the presence of ADP-ribosylated actin may occur at barbed ends. Similar results were also observed with the ADP-ribosylated non-muscle actin and component I of C_2 toxin (Weight et al. 1989). Therefore, the ADP-ribosylated actin behaves like a capping protein to inhibit further polymerization of actin monomers at the barbed ends. The actin monomers released from the pointed ends, which are not capped with ADP-ribosylated actins, are supplied as the substrates for further ADP-ribosylation (Fig. 2). These molecular events in cells exposed to botulinum C_2 toxin or *C. perfringens* iota toxin seem to be the causes of the disorganization of microfilament networks.

It has been reported that the actin in gelsolin–actin or gelsolin–actin–actin complexes is ADP-ribosylated in vitro by both botulinum C_2 and *C. perfringens* iota toxins (Wille et al. 1992). It was also demonstrated that the actin of the gelsolin–actin complex in intact human neutrophils was ADP-ribosylated by C_2 toxin (Just et al. 1993). These findings indicate that the ADP-ribosylation site (Arg-177) of actin is not masked in the actin of gelsolin–actin complexes, and the complexes are additional target molecules for toxins in the cells. Although the nucleating activities of the gelsolin–ADP-ribosylated actin and gelsolin–ADP-ribosylated actin–actin complexes were indistinguishable from that of the gelsolin–actin complex, actin polymerization in the presence of the gelsolin–ADP-ribosylated actin complexes was significantly retarded compared with polymerization nucleated by the gelsolin–actin complex

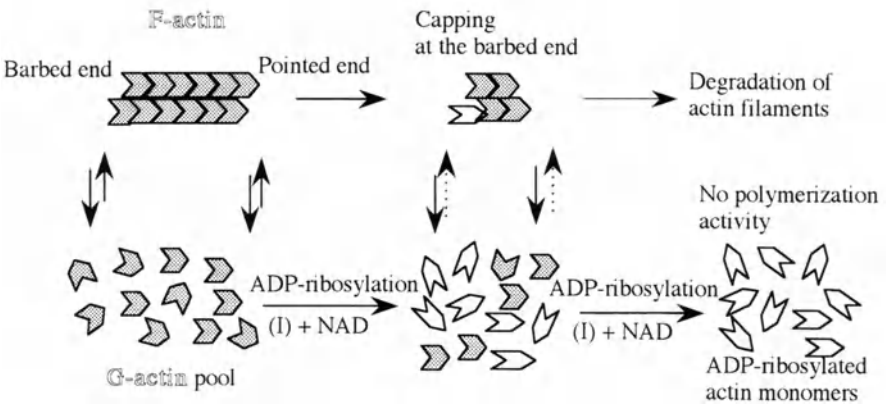

Fig. 2. Model for the degradation of actin filaments by adenosine diphosphate (*ADP*)-ribosylation of actin monomers with component I of botulinum C2 toxin. Firstly, polymerization of actin proceeds at both barbed and pointed ends by incorporating actin monomers (G-actin), although polymerization at barbed ends is faster than that at pointed ends. The G-actins ADP-ribosylated by component I of botulinum C2 toxin then bind to the barbed ends of actin filaments in a capping-protein-like manner, thereby inhibiting further polymerization at the fast-growing end of F-actin. Lastly, and in addition, the modified actins are incapable of polymerization (Aktories 1990)

(Wille et al. 1992). Thus, the interaction of ADP-ribosylated actins with actin-binding proteins (including gelsolin and possibly others) is another mechanism of the cytopathic effects of the actin–ADP-ribosylating toxins.

C. *C. Perfringens* Iota Toxin

I. Actin–ADP-Ribosylating Toxin of *C. Perfringens* Type E

C. perfringens is, at present, divided into five types (A–E) depending on their ability to produce four toxins: α, β, ι and ε. Iota toxin is produced only by *C. perfringens* type E strains; it is produced along with α toxin (which is a single polypeptide of 42.5 kDa), phospholipase C, sphingomyelinase and hemolytic and lethal activities. *C. perfringens* type E has been implicated in enteric disease in calves, lambs and rabbits (Bosworth 1943; Ross et al. 1949). It had been known that iota toxin produced by *C. perfringens* type E is immunologically related to the toxin produced by *C. spiroforme* (Borriello and Carman 1983). In 1986, Stiles and Wilkins found the two protein components of iota toxin by immunoelectrophoresis in the culture supernatant of *C. perfringens* type E; they designated these components i_a and i_b (Stiles and Wilkins 1986a). Moreover, they demonstrated that i_a and i_b of iota toxin were not linked by covalent or noncovalent bonds; these individual components showed little or

no activity (STILES and WILKINS 1986a). When these two proteins were combined, synergistic effects on mouse lethality and guinea pig dermonecrotic activity were observed, indicating that the molecular structure of iota toxin is analogous to that of botulinum C_2 toxin. At that time, however, it had not yet been revealed that i_a of iota toxin is actin-specific ADP-ribosyltransferase. In 1987, SIMPSON et al. reported evidence that one of iota toxin components, i_a, is an enzyme with mono-ADP-ribosylating activity, with homo-poly-l-arginine as the substrate (SIMPSON et al. 1987). Thereafter, SCHERING et al. demonstrated that the ADP-ribosyltransferase activity of i_a is specific for actin and modifies both muscle and non-muscle actins (SCHERING et al. 1988).

II. Molecular Structure and Function

STILES and WILKINS reported purification procedures for the two protein components (i_a and i_b) of iota toxin produced by *C. perfringens* type-E strain NCIB 10748 (STILES and WILKINS 1986b). These two components are non-linked proteins and are synergistic in eliciting the biological activity of the toxin; either component alone was inactive in terms of lethality in mice and dermonecrotic activity in guinea pigs, whereas the combination of the two was active. The molecular sizes of i_a and i_b, as determined by SDS-PAGE, were 47.5 kDa and 71.5 kDa, respectively, and each of them cross-reacted with antiserum to *C. spiroforme*. These two proteins of iota toxin did not show any synergistic effect with the two components of botulinum C_2 toxin; i.e., neither the combination of i_a of iota toxin and the component II of C_2 toxin nor that of i_b of iota toxin and the component I of C_2 toxin was lethal to mice, implying that the primary structure of *C. perfringens* iota toxin is quite different from that of botulinum C_2 toxin, although the structure–function relationship of iota toxin mimics that of C_2 toxin.

In 1988, SCHERING et al. reported that the target molecule of i_a of iota toxin is actin (SCHERING et al. 1988). In contrast to C_2 toxin of *C. botulinum*, however, i_a ADP-ribosylated both muscle and non-muscle actins, whereas component I of C_2 toxin ADP-ribosylated only non-muscle actin, as described in Sect. B. Five different actin isoforms – α-skeletal-muscle actin, α-cardiac-muscle actin, gizzard γ-smooth-muscle actin, spleen β/γ-cytoplasmic actin and aortic α/γ-smooth-muscle actin (this compound contained 77% α isoform and 23% γ isoform) – were prepared and compared with respect to the substrate specificity for iota and C_2 toxins (MAUSS et al. 1990). The i_a component of iota toxin modified all of these actin isoforms, whereas component I of C_2 toxin modified gizzard γ-smooth-muscle and spleen β/γ-cytoplasmic actins and the γ-isoform of aortic α/γ-smooth muscle actin. The modification of actin by i_a resulted in the inhibition of the polymerization activity of actin. Cytoplasmic actin in intact chicken embryo cells exposed to iota toxin was ADP-ribosylated, and the modification was counteracted with component I of C_2

toxin. Pretreatment of the cells by C_2 toxin resulted in marked reduction of ADP-ribosylation of cytoplasmic actin by iota toxin, suggesting that the target site on the actin molecule is identical for both component I of C_2 toxin and i_a of iota toxin. Since the ADP-ribosylation site for C_2 toxin was identified as Arg-177 of non-muscle actin, and since this site is identical for muscle and non-muscle actins, both iota toxin and C_2 toxin were presumed to modify this amino acid. Thereafter, the ADP-ribosylation site of skeletal-muscle actin (α-actin) by iota toxin was identified as Arg-177, which is the same as the target of botulinum C_2 toxin (VANDEKERCKHOVE et al. 1987).

In 1993, iota-toxin genes of *C. perfringens* type E strain NCIB 10748 were cloned, and the details of the molecular characteristics were reported by PERELLE et al (1993). The i_a gene encoded 454 amino acids (52.314 kDa) containing a signal peptide of 41 amino acid residues at the N-terminal end (matured i_a is composed of 413 amino acids, 47.586 kDa). The i_a sequence reported in 1993 was incorrect, and the correct sequence was reported in 1995 (PERELLE et al. 1995). The i_b gene encodes 875 amino acids (98.467 kDa) containing a signal peptide of 39 residues at the N-terminal end (matured i_b was 836 residues, 94.013 kDa). The trypsin-activated form of i_b appeared to be composed of amino acid residues from Ala-212 to Asn-875 (664 amino acids, 80.890 kDa; PERELLE et al. 1993). In the primary structure of i_b of iota toxin, the hydrophobic sequence from Leu-292 to Ser-309, which possibly forms a transmembrane segment across the cell membrane, was determined. I_b is required for entry of i_a into the cytosol, and i_b must undergo limited proteolysis to be functionally active. The cloning of the gene encoding component II of C_2 toxin revealed a similar transmembrane segment from Met-260 to Ser-277 (KIMURA et al. 1998). Thus, iota toxin shares a comparable structure and mode of action with botulinum C_2 toxin.

In 1996, VAN DAMME et al. reported that the active site for ADP-ribosylation activity of iota toxin is located at Glu-378, which seems to be pivotal for catalysis, because the residue is cross-linked to nicotinamide adenine dinucleotide (NAD) by UV-irradiation; this is accompanied by inhibition in ADP-ribosylation activity (VAN DAMME et al. 1996). They suggested that the spaced biglutamic acid sequence, which was found in iota toxin at Glu-378 and Glu-380, participates in the catalysis of ADP-ribosylation activity, as occurs in various transferases, including bacterial toxins [*Staphylococcus aureus* transferase (epidermal cell differentiation inhibitor), *Vibrio cholerae* toxin, *Escherichica coli* heat labile toxin and *Bordetella purtussis* toxin]. PERELLE et al. also reported that the active site of i_a for ADP-ribosyltransferease is located in the C-terminal region of the protein (PERELLE et al. 1996). Site-directed mutagenesis of amino acid residues (Arg-295-Lys, Glu-378-Ala, Glu-380-Asp and Glu-380-Ala) in the C-terminus of the toxin induced a drastic decrease in both ADP-ribosylation and cytotoxic activities. This is consistent with the results of labeling iota toxin with NAD^+ by UV-irradiation, as described above (DAMME et al. 1996). The conserved NAD^+-binding site in component I of C_2 toxin was found to be composed of residues Glu-331 to Try-336 (FUJII et al.

1996). However, the homology between component I and i_a was reported to be only about 10% (this was incorrectly calculated, and the homology recalculated with the data of D63902 and X73562 in the DNA Data Bank of Japan is 36%), whereas that between component II and i_b was 39% (FUJII et al. 1996; KIMURA et al. 1998)

D. *C. Spiroforme* Toxin

I. Actin–ADP-Ribosylating Toxin of *C. Spiroforme*

C. spiroforme is an enteric pathogen of weaning rabbits and causes enterotoxin-mediated diarrhea, which is possibly a common cause of mortality among rabbits. BORRIELLO and CARMAN reported the detection of iota-like toxin and the isolation of *C. spiroforme* from cecal contents of rabbits suffering from spontaneous or antibiotic-associated diarrhea (BORREIELLO and CARMAN 1983). As described in Sect. C, in 1986, STILES and WILKINS demonstrated that iota toxin elaborated by *C. perfringens* was cross-neutralized with antiserum to *C. spiroforme* (STILES and WILKINS 1986b). Moreover, they reported that *C. perfringens* type E and *C. spiroforme* both produce two protein components, i_a and i_b. These two components were purified from the culture supernatant of *C. perfringens* type E strain NCIB 10748 by isoelectric focusing. The biological activity of the toxin increased when the two purified protein components were combined, as described in Sect. C. These findings indicate that *C. spiroforme* also produces iota-like toxin, the molecular structure and function of which is analogous to that of iota toxin of *C. perfringens*. The two purified proteins of *C. spiroforme* were characterized in detail, and one of the two components was found to be actin-specific ADP-ribosyltransferase (POPOFF and BOQUET 1988; POPOFF et al. 1989).

II. Molecular Structure and Function

In 1988, POPOFF and BOQUET reported immunological cross-reactions among purified ADP-ribosyltransferases of *C. spiroforme* strain 246 and *C. perfringens* type E strain NCIB 10748, the enzymatic component of *C. difficile* strain C196 (DCT; Sect. D) and component I of botulinum C_2 toxin. The ADP-ribosyltransferases purified from *C. spiroforme*, *C. perfringens* and *C. difficile* cross-reacted with each other in an immunoblotting assay; only component I of botulinum C_2 toxin did not cross-react. All of these purified components ADP-ribosylated actin prepared from the lysate of *Xenopus* oocytes. *C. spiroforme*, in addition to the enzyme component (designated as Sa), produced another protein component, Sb, which showed a synergistic effect on lethality in mice and cytotoxicity in cultured cells when combined with Sa (POPOFF et al. 1989, SIMPSON et al. 1989). They also examined the interchangeability of the enzyme components with the binding components of the four clostridial

strains. As with immunological effects, only component I of C_2 toxin is exceptional, although the two components from the other three clostridial strains were interchangeable with their counterparts from the toxins. All of the combinations (i_a and Sb, CDT and Sb, Sa and i_b, CDT and i_b) were cytotoxic to cultured Vero cells. The results indicate that *C. spiroforme* produces a "binary toxin" whose molecular structure and function are similar to those of *C. perfringens* type E iota toxin (Simpson et al. 1989). The molecular weights of the two proteins, Sa and Sb, as determined by SDS-PAGE, were 44 kDa and 92 kDa, respectively, while that of Sb activated by trypsin was 76 kDa (Popoff et al. 1989). As demonstrated with the two components (I and II) of botulinum C_2 toxin, these components alone were neither lethal to mice nor cytotoxic to cultured cells (Simpson et al. 1989).

E. *C. Difficile* Toxin

I. Actin–ADP-Ribosylating Toxin of *C. Difficile*

C. difficile is the pathogenic organism in pseudomembranous colitis and antibiotic-associated diarrhea (Rehg and Lu 1981; Carmen and Evans 1984) and produces two large-molecular-weight, single-polypeptide toxins, toxins A (308 kDa) and B (270 kDa; Barroso et al. 1990; Sauerborn and Von Eichel-Streiber 1990). Toxin A is a lethal enterotoxin and induces intestinal inflammation, causing hemorrhagic diarrhea, whereas toxin B is not enterotoxigenic but is potently cytotopathogenic to cultured cells. These toxins are not ADP-ribosyltransferases but are monoglucosyltransferases that covalently modify the Rho family by uridine-diphosphate–glucose-dependent glycosylation at threonine 37 and result in inactivation of Rho proteins, thereby inducing disassembly of cytoskeletal actin filaments (Just et al. 1995a, 1995b). In addition to toxins A and B, it has been reported that one of 15 *C. difficile* strains examined produced actin-specific ADP-ribosyltransferase, designated CDT (Popoff and Boquet 1988).

II. Molecular Structure and Function

In 1988, Popoff and Boquet reported an unique strain of *C. difficile*, strain CD196, which produced only an actin-specific ADP-ribosyltransferase; the microinjection of culture supernatant of the strain into [^{32}P]NAD-loaded *Xenopus laevis* oocytes resulted in ADP-ribosylation of cytoplasmic actin (Popoff and Boquet 1988; Popoff et al. 1988). The purified enzyme component, designated CDT, weighed 43 kDa, as determined by SDS-PAGE, and was not immunologically related to *C. difficile* toxins A or B or with components I or II of botulinum C_2 toxin. CDT was not cytotoxic to cultured Vero cells and was not lethal to mice by intraperitoneal injection (Popoff et al. 1988).

Moreover, CDT was neither cytotoxic nor lethal, even when combined with component I or II of botulinum C_2 toxin. However, the ADP-ribosylation of cytoplasmic actin by CDT in lysates of Vero cells was counteracted by botulinum C_2 toxin; exposure of the cells to C_2 toxin depleted the actin substrate available for modification by CDT. In immunoblotting assays with anti-CDT serum, CDT immunologically cross-reacted with a 43-kDa protein in the culture supernatant of *C. perfringens* type E, which is known to produce iota toxin, an actin-specific ADP-ribosyltransferase. In combination with the binding component of either iota toxin or *Spiroforme* toxin, CDT showed cytotoxicity to Vero cells and lethality in mice, whereas it did not do so when combined with component II, the binding component of botulinum C_2 toxin. These results imply (1) that CDT is ADP-ribosyltransferase, which is antigenically similar to *C. perfringens* iota toxin, and (2) that *C. difficile* strain CD196 produces only the enzyme component corresponding to component I of botulinum C_2 toxin. Thus, CD196 seemed to be an exceptional strain producing no binding component of "binary toxin". This puzzle was solved in 1997 by PERELLE et al. Using an immunoblotting method, they detected two proteins – the enzyme and binding components (designated as CDTa and CDTb, respectively) of *C. difficile* toxin – in 40-fold-concentrated culture supernatants of strain CD196. Moreover, they demonstrated that the CDT locus of *C. difficile* strain CD196 contains two genes, *cdtA* and *cdtB*, which encode CDTa and CDTb, respectively. In addition, they showed that the lower production levels of both components are due to lower transcription levels and, probably, to the lower stabilities of their mRNAs. The levels of the transcription products of the genes for *cdtA and cdtB* were approximately 30- to 60-fold lower than those of *C. perfringens* type E iota toxin.

The antigenicity of CDTb is similar to those of both Sb and i_b but not that of component II of C_2 toxin. Similarly, CDTa cross-reacts immunologically with Sa and i_a but not with component I of C_2 toxin (PERELLE et al. 1997). The molecular weights of CDTa and CDTb, determined by SDS-PAGE, are 43 kDa and 75 kDa, respectively. The morphological changes in cultured cells exposed to concentrated culture supernatants of CD196 are very similar to those caused by botulinum C_2 toxin, iota toxin and *Spiroforme* toxins: the rounding of the cells, as detected by light microscopy, and the disassembly of microfilaments, detected by staining with fluorescently labeled phalloidin. Trypsinization of CDTb is required to achieve full activity of CDT toxin. The apparent molecular size of the activated CDTb is 73 kDa in SDS-PAGE. The amino acid sequences of CDTa and CDTb (deduced from gene cloning) are 81% and 84% identical to those of i_a and i_b of iota toxin, respectively.

Though not yet examined, CDT probably modifies muscle actin, because the toxin has molecular characteristics very similar to those of iota toxin, which ADP-ribosylates both non-muscle and muscle actins. Thus, CDT is another "binary toxin" of actin-specific ADP-ribosyltransferease and is apparently involved in the pathogenesis of *C. difficile* infection.

Table 2. Actin–adenosine diphosphate (ADP)-ribosylating toxins

Toxin producer	Toxin components (molecular weight)[a]	Substrate specificity	Antigenically common?
C. botulinum types C and D	C_2 toxin[b]: component I (55 kDa)	β/γ-Actin	Not with i_a, Sa and CDTa
	C_2 toxin[b]: component II (105/88 kDa)		Not with i_b, Sb and CDTb
C. perfringens type E	Iota toxin: i_a (47.5 kDa)	α- and β/γ-actins	Not with (I)[c] but with i_a, Sa and CDTa
	Iota toxin: i_b (71.5 kDa)		Not with (II)[c] but with i_b, Sb and CDTb
C. spiroforme	Spiroforme toxin: Sa (44 kDa)	β/γ- and possibly α-actins[d]	Not with (I) but with i_a, Sa and CDTa
	Spiroforme toxin: Sb (92/76 kDa)		Not with (II) but with i_b, Sb and CDTb
C. difficile	CDT[e]: CDTa (43 kDa)	β/γ- and possibly α-actins[d]	Not with (I) but with i_a, Sa and CDTa
	CDT[e]: CDTb (75/73 kDa)		Not with (II) but with i_b, Sb and CDTb

[a] Molecular size was determined by sodium dodecyl sulfate polyacrylamide-gel electrophoresis. The weights of component II, Sb and CDTb are given as the weight of the component when not activated by trypsin/the weight of the component when activated by trypsin. See the text for a discussion of the molecular weights deduced from gene cloning.

[b] See Sect. B.II. for a discussion of the heterogeneity (depending on the strain) of the two components of C_2 toxin in biological activity and molecular size.

[c] (I) and (II) are short for components I and II of C_2 toxin, respectively.

[d] Only cytoplasmic actin was examined for ADP-ribosylation with these toxins. Therefore, it has not yet been reported whether all of these actin isoforms are modified or not. However, the immunological cross-reaction and the homology in amino acid sequence support this substrate specificity.

[e] *Clostridium difficile* toxin?

F. Concluding Remarks

Most bacterial toxins are enzymes that modify, in most cases, a key protein in a living system of eukaryotes. Like all other types of protein catalyst, the toxin enzyme has substrate specificity and catalyzes a chemical reaction at an extremely high rate. In addition, as we learn from basic enzymology, the enzyme molecules themselves ideally are not changed after participating in the reaction and, therefore, can function many times. All of these characteristics of the toxin enzyme explain the rapid, specific and serious damage to cells exposed to the toxin. Paradoxically, the substrate specificity of a bacterial toxin is an useful tool to analyze the specific role of the substrate protein at a molecular level in the cell. The bacterial toxins are sometimes considered

to be specialized molecules that cause an unusual cellular response. However, in any field of science, our ideas always change from specific ones to general ones and vice versa. Therefore, the fundamental principles of cell biology, I hope, could be revealed by the toxinology of clostridial actin-specific ADP-ribosyltransferases. The molecular structures and functions of clostridial actin-specific ADP-ribosyltransferases are summarized in Table 2.

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CHAPTER 13

Molecular Biology of Actin-ADP–Ribosylating Toxins

M.R. Popoff

A. Introduction

The actin cytoskeleton is the target of a large number of toxins. The actin network controls the shape and spatial organization of the cell and contributes to many cell functions, such as movement, cytokinesis, endocytosis, exocytosis and control of intercellular junctions. Its disorganization by toxins leads to important cell dysfunctions and induces the severe lesions which are found in the associated pathologies. Some toxins modify enzymatic regulatory proteins of actin polymerization (Rho family), including: C3 enzyme, which specifically adenosine diphosphate (ADP)–ribosylates Rho (Chap. 11); the large clostridial toxins [*Clostridium difficile* ToxA and ToxB, *C. sordellii* heat-labile toxin (LT) and hemorrhagic toxin, and *C. novyi* Toxα], which monoglucosylate various p21 G-proteins (Chap. 14); and cytotoxic necrotizing factor from *Escherichia coli* and dermonecrotizing toxin from *Bordetella bronchiseptica* (Chap. 13), which catalyze the deamidation of Gln-63 in Rho protein. Modification by ADP–ribosylating or glucosylating toxins traps the Rho family proteins in their inactive forms, leading to depolymerization of the actin filaments in cells. In contrast, the deamidation of Gln-63 impairs the Rho guanosine triphosphate (GTP)ase activities, and Rho is permanently active (Flatau et al. 1997; Schmidt et al. 1997). This increases the actin filaments and ruffles and blocks the cytokinesis. Other toxins act directly on the actin monomers by ADP–ribosylation, which is a common mechanism of action for many toxins (Chap. 2); thereby, they cause a complete disorganization of the actin filaments. These toxins, which are called actin-ADP–ribosylating toxins, are produced by bacteria from the *Clostridium* genus.

The actin-ADP–ribosylating toxins are binary toxins and share a common structure consisting of two independent protein chains (binding and enzymatic components). The binding component is involved in the recognition of a cell-surface receptor and mediates the internalization of the enzymatic component into the cytosol. The enzymatic component modifies the actin monomers by ADP–ribosylation at Arg-177, leading to the inhibition of actin polymerization and the disassembly of actin filaments (Chap. 18). The other ADP–ribosylating toxins, such as diphtheria toxin (DT) and cholera toxin (CT), are also organized into different functional domains. The special feature of the binary toxins is that the enzymatic domain is localized on a distinct protein

chain that is not linked (by either covalent or non-covalent bonds) to the binding component. This review is focussed on current data about the genetic aspects of the actin-ADP–ribosylating toxins.

B. Bacteria Producing Actin-ADP–Ribosylating Toxins

The bacteria that produce actin-ADP–ribosylating toxins and their main associated pathologies are listed in Table 1.

I. *C. Botulinum*

C2 toxin is produced by *C. botulinum* type-C and -D strains, which are the causative agents of botulism in diverse animal species and, rarely, in humans. These *C. botulinum* strains also produce botulinum neurotoxins C1 and D, respectively, and the C3 enzyme. The C2 toxin is not involved in the neurologic disorder of botulism, but it is probably responsible for the necrotic and hemorrhagic lesions of the intestinal wall which are occasionally observed during the autopsies of animals that die of botulism, particularly in avians (Eklund and Dowell 1987). Experimentally, C2 toxin is dermonecrotic and induces hemorrhagic enteritis in mice (Ohishi and Odagiri 1984).

II. *C. Perfringens*

C. perfringens is widespread in the environment and is divided into five toxinotypes (A, B, C, D and E) according to the major toxins, which are lethal for mice. *C. perfringens* E is characterized by the production of Iota and Alpha toxins as major toxins. This microorganism type is an occasional cause of diar-

Table 1. Actin-adenosine diphosphate (ADP)–ribosylating toxin-producing Clostridia-associated pathology and hosts

Bacterial species	Actin-ADP–ribosylating toxin	Other toxins	Pathology	Host
C. botulinum C	C2	Neurotoxin C1, C3	Botulism	Birds, minks, other animals
C. botulinum D	C2	Neurotoxin D, C3	Botulism	Cattle, other animals
C. perfringens E	Iota	Alpha	Enterotoxemia	Calves, other young animals
C. spiroforme	*C. spiroforme toxin*	–	Diarrhea, death	Rabbit, human
C. difficile	CDT	ToxA, ToxB	Diarrhea, colitis	Humans, rodents

rhea and enterotoxemia in calves and, more rarely, in other young animals. The enteric clostridial affections, including *C. perfringens* enterotoxemia, result from colonization (by Clostridia) of the digestive tracts of hosts with compromised intestinal flora, and from the production of toxins. The most common predisposing factors are the use of antibiotics, and weaning. The toxins produced in the intestinal content are responsible for the symptoms and lesions. The Iota toxin is dermonecrotic and lethal for mice when injected intraperitoneally (McDonell 1986).

III. *C. Spiroforme*

C. spiroforme, so called because of its spirally coiled chains of curved rods, is commonly found in the digestive tracts of rabbits, and it is a major cause of diarrhea and death in this animal species (Carman et al. 1997). The *C. spiroforme* toxin causes fluid accumulation when injected into ligated sections of rabbit ileum. The intestinal submucosa is thickened by an inflammatory exudate, and the mucosal epithelium shows varying degrees of necrosis, leading to ulcerations and even to diffuse mucosal denudation (Carman et al. 1997). One case of colitis and diarrhea in a leukemic human has been reported (Baudieri et al. 1986).

IV. *C. Difficile*

C. difficile is the well-documented agent of pseudomembranous colitis and diarrhea following antibiotic treatment. The major toxins synthesized by *C. difficile* are ToxA and ToxB (Chap. 15). It has been found that a *C. difficile* strain (CD196) produces an actin-specific ADP–ribosyltransferase (CDT) immunologically related to the Iota toxin and distinct from ToxA and ToxB, which are also synthesized by this strain. However, a binding component for CDT was not evidenced (Popoff et al. 1988). Genetic analysis and DNA sequencing showed that CD196 contains both enzymatic (CDTa) and binding-component (CDTb) genes and that they are related to the corresponding genes of the Iota toxin from *C. perfringens* (Perelle et al. 1997a).

The exact role of CDT in the natural *C. difficile* diseases remains to be determined. CD196 was isolated from a patient with severe pseudomembranous colitis (Popoff et al. 1988). In a short series, 12.5% of *C. difficile* strains isolated from patients with enteritis contained CDT genes in addition to those coding ToxA and ToxB (Perelle et al. 1997a). This suggests that CDT could be an additional virulence factor and that CDT-producing strains could be involved in severe *C. difficile* afflictions.

C. Families of Actin-ADP–Ribosylating Toxins

The clostridial actin-ADP–ribosylating toxins share the same structure, consisting of two independent protein components (Tables 1, 2). However, two

Table 2. Immunological cross-reactions between the binding components of the Iota and C2 families by enzyme-linked immunosorbent assay

	PA	Ib	Sb	C2-II
Anti-PA	400000	<100	<100	<100
Anti-Ib	1200	1600000	410000	<100
Anti-Sb	100	102000	102000	<100
Anti-C2-II	350	2400	1000	16000

PA, protective antigen.

families of actin-ADP–ribosylating toxins can be distinguished on the basis of their immunological relatedness and biological activites, one corresponding to the C2 toxins from *C. botulinum* (C2-toxin family), and the other to the *C. perfringens* Iota toxin, *C. spiroforme* toxin and *C. difficile* transferase (Iota-toxin family).

I. C2-Toxin Family

The C2 toxin family is heterogeneous. Three groups of C2 toxins from *C. botulinum* C and D strains have been identified according to their molecular weights (MWs) and immunological cross-reactions (OHISHI and OKADA 1986).

The electrophoretic mobilities of the enzymatic component (C2I) from the three groups are approximately the same (~49kDa). C2I from the three different groups share common epitopes, but a partial immunological reaction was observed between C2I from groups I and II (OHISHI and OKADA 1986).

The binding components (C2II) from the three groups are dissimilar in size (84, 69 and 80kDa for the mature proteins of groups I–III, respectively) and are immunologically heterogeneous, as tested by immunoprecipitation (OHISHI and HAMA 1992). The heterogeneity has been confirmed by polymerase chain reaction (PCR) amplifications (KIMURA et al. 1998). Moreover, the biological activities (vascular permeability and cell rounding) were different for the C2 toxins from the three groups (OHISHI and OKADA 1986; OHISHI and HAMA 1992). The vascular-permeability activity of C2 toxins from group II, tested by intradermal injection in rabbit, was higher than that of C2 toxins from groups I and III. C2 toxin from group III produced very low vascular-permeability activity. Combinations of C2I from group III and C2II from group I formed toxins with low activity, whereas combination of C2I from group I with C2II from group II or III reconstituted active toxins. C2II of group III was more efficient than C2II of group II in forming active toxin with C2I of group I. This indicates that component II of group III is as active as those of groups I and II, and component I of group III is possibly inactive (OHISHI and OKADA 1986; OHISHI and HAMA 1992).

II. Iota-Toxin Family

The enzymatic and binding components of the Iota-toxin family produced by *C. perfringens*, *C. spiroforme* and *C. difficile* have different sizes (Figs. 1, 2) but are immunologically related. The antibodies raised against the enzymatic components of Iota toxin (Ia), *C. spiroforme* toxin (Sa) and CDTa recognize indistinctly each enzymatic component by Western blotting (Fig. 3). Cross-reactions between Ib and Sb have also been observed by Western blotting and enzyme-linked immunosorbent assay (ELISA; Table 2). Heterogeneity between toxins produced by individual strains of the same *Clostridium* species remains to be determined. Moreover, the binding and enzymatic components of the Iota family can be interchanged to form toxins fully active in cell rounding and mice lethality. For example, the binding components of Iota toxin (Ib) and *C. spiroforme* toxin (Sb) can mediate the internalization and cytotoxicity of CDTa (Popoff and Boquet 1988).

III. Relatedness Between C2-Toxin and Iota-Toxin Families

The toxins from the two families are not, or are only weakly, immunologically related (Fig. 3; Table 2), and no functional complementation was evidenced between the components of each family (Table 3). Thus, the binding components of the Iota-toxin family cannot translocate the enzymatic component of C2 toxin into host cells; similarly, the binding component of C2 toxin is inefficient in permitting the translocation of the enzymatic proteins of the Iota

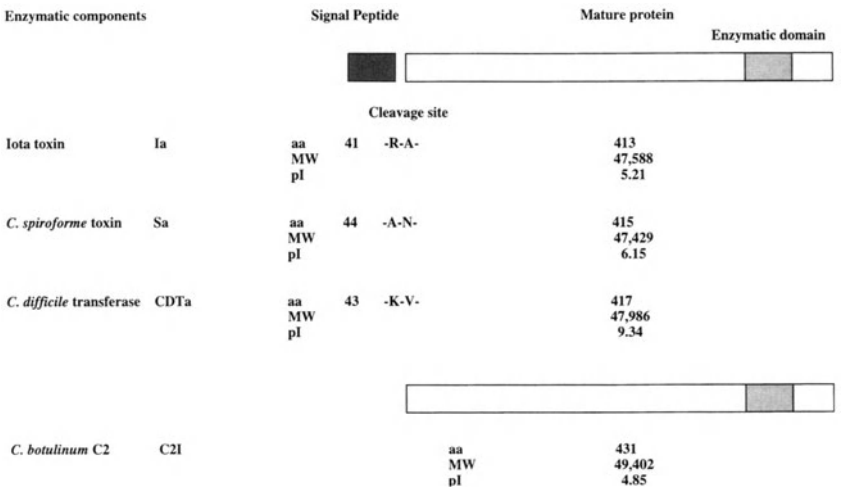

Fig. 1. Structure and domain organization of the enzymatic components of actin-adenosine diphosphate–ribosylating toxins

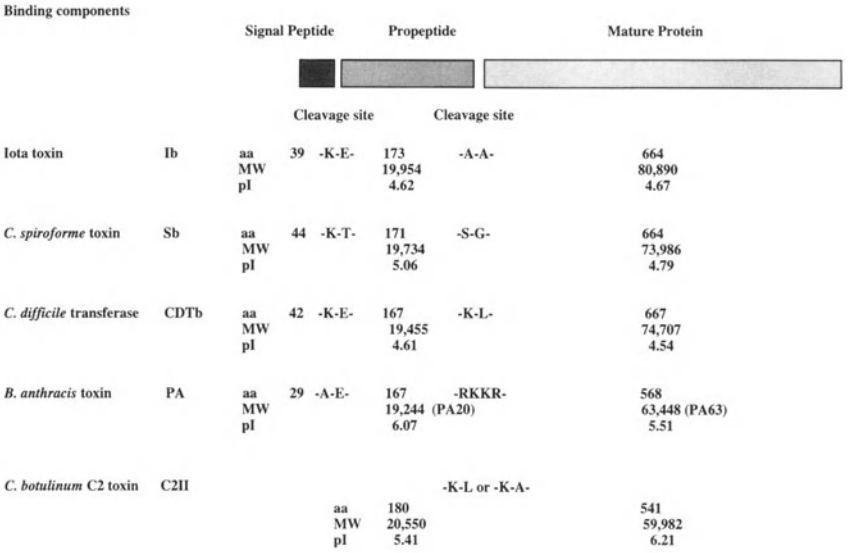

Fig. 2. Structure and domain organization of the binding components of actin-adenosine diphosphate–ribosylating toxins

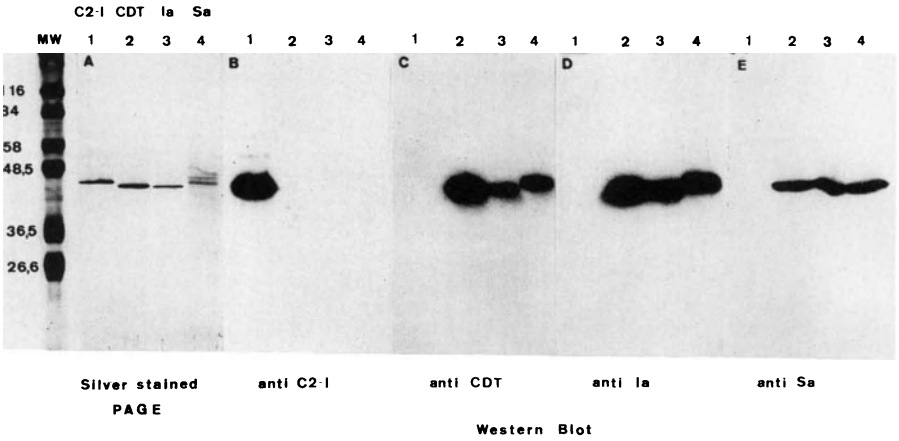

Fig. 3. Immunological relatedness of the enzymatic components of actin-adenosine diphosphate–ribosylating toxins as tested by Western blotting (POPOFF et al. 1988, 1989). **A** Silver-stained polyacrylamide gel electrophoresis of C2I (1), CDTa (2), Ia (3) and Sa (4). **B–E** Western blotting with antibodies anti-C2I (B), anti-CDTa (C), anti-Ia (D) and anti-Sa (E)

Table 3. Comparison of the structure and properties of toxins from Iota and C2 families

	Iota family	Inter-family comparison	C2-toxin family
Toxins	*C. perfringens* Iota toxin *C. spiroforme* toxin *C. difficile* (CDT)		C2 toxins of *C. botulinum* C and D (three groups)
Amino acid identity	80–85%	31–40%	?[a]
Immunological cross-reaction	High	Weak	High
Functional complementation between enzymatic and binding proteins	Yes	No	Yes
Cell-surface receptor	Unidentified	Different	Unidentified
Intracellular target of the enzymatic components	All actin isoforms		Cellular actin isoforms
Enzymatic reaction		ADP–ribosylation at Arg-177	

ADP, adenosine diphosphate.
[a] C2-toxin sequence was determined from only one *C. botulinum* strain.

family (Popoff and Boquet 1988; Considine and Simpson 1991). Other differences between the two families concern the recognition of different cell-surface receptors by the binding components, and the mode of entry into cells (Table 3).

The binary toxins are internalized into cells by receptor-mediated endocytosis (Simpson 1989). The cell receptors have not yet been identified. C2II may recognize a receptor related to those of growth factors (Fritz et al. 1995). No competition was observed between C2II and Ib in internalizing either C2I or Ia, indicating that C2 and Iota toxins recognize different cell-surface receptors (unpublished). Furthermore, a Chinese hamster ovary (CHO) mutant cell line resistant to the C2 toxin was isolated. The ADP–ribosylation of G-actin by C2I was not impaired in lysates of mutant cells. However, the binding of C2II to the resistant cells was drastically decreased, indicating that the mutation affected the C2II receptor. Interestingly, the C2-resistant CHO cells remained sensitive to Iota toxin, confirming that the C2II receptor is different from that of Ib (Fritz et al. 1995).

The cytopathic effects of C2 toxin are blocked by agents preventing endocytic pathway acidification (chloroquine, bafilomycin A1 and monensin),

whereas the cytotoxicity induced by Iota toxin and *C. spiroforme* toxin are not modified by these agents (Popoff et al. 1989; Simpson 1989; Gibert et al. 1997; Perelle et al. 1997b). It has been found that *C. spiroforme* toxin enters cells by coated-pit vesicles, since its cytotoxicity is blocked by potassium depletion, which impairs the clathrin assembly. The translocation of Sa into the cytosol seems to take place in the cell periphery. Therefore, a temperature of 15°C, which inhibits the traffic from endosomes to the trans-Golgi network, does not affect the *C. spiroforme* toxin effects (Popoff et al. 1989). This indicates that the Iota toxins are routed in a different way than via the acidic endosomes used by C2 toxin. Furthermore, the intracellular targets of the enzymatic components are different. Iota toxin is able to modify all the actin isoforms, whereas the specificity of C2 toxin is restricted to non-muscular actin (Mauss et al. 1990; Table 3).

D. Actin-ADP–Ribosylating Toxin Genes and Predicted Molecules

I. Iota-Toxin Genes and Iota-Toxin Proteins

The Iota-toxin genes were the first genes of actin-ADP–ribosylating toxins to be characterized. The DNA region of *C. perfringens* E strain NCIB10748 encoding the Iota-toxin structural genes has been cloned and sequenced using degenerated oligonucleotides derived from protein sequencing (Perelle et al. 1993). Two genes (*iap* and *ibp*) localized in the close vicinity encode Ia and Ib components, respectively.

The coding regions of *iap* and *ibp* genes predict a protein 454 amino acids long for Ia and a protein 875 amino acids long for Ib. The 41 N-terminal amino acids of Ia and the 39 corresponding amino acids of Ib have the features of bacterial signal sequences, including the presence of charged N-terminal residues, a long hydrophobic core followed by charged residues, a turn residue and a cleavage site for signal peptidase. Therefore, the mature Ia consists of 413 amino acids (Fig. 1). The predicted MW (47 588 Da) and pI (5.01) are in agreement with those determined experimentally (MW 47 500 Da and pI 5.2; Stiles and Wilkins 1986b; Perelle et al. 1993). The recombinant *iap* gene expressed in *E. coli* yielded a protein migrating at 44 kDa; it was recognized by anti-Ia antibodies and exhibited ADP–ribosyltransferase activity with actin as substrate (Perelle et al. 1993).

The secreted Ib encompasses 836 amino acids and corresponds to the Ib precursor. The mature Ib (664 amino acids) results from the proteolytic cleavage of the N-terminal propeptide (172 amino acids) from the precursor form (Fig. 2). The predicted MW (80 890 Da) and pI (4.67) differ from those found for native Ib (67 000 Da and pI 4.2; Stiles and Wilkins 1986b). Recombinant Ib produced in *E. coli* is recognized by anti-Ib antibodies, but it is not processed in its mature form (Perelle et al. 1993).

II. *C. Spiroforme* Toxin and CDT Genes

The genes of *C. spiroforme* toxin and CDT have been cloned and sequenced from one representative bacterial strain of each species. The toxins of the Iota family are immunologically related, suggesting that they have a similar sequence. Therefore, the cloning of the homologous genes was facilitated by the fact that Iota toxin recombinant clones, which were used as probes, hybridized with DNA from *C. spiroforme* and *C. difficile*. The genes (at least those that were characterized) that code the toxin components of the Iota family have approximately the same size and show the same organization. The two genes encoding the enzymatic and binding components are in the close vicinity, and they are transcribed in the same orientation. They are separated by 40 non-coding nucleotides in *C. perfringens* E, 41 in *C. spiroforme* and 52 in *C. difficile*. The gene encoding the enzymatic component is localized upstream from that encoding the binding component. A consensus ribosome-binding site (GGAGG) is found upstream from each open reading frame. An inverted repeat able to form a hairpin-loop structure and probably corresponding to a Rho-independent terminator sequence is lying downstream from the binding component gene in *C. difficile* ($\Delta G = -16.1$ kcal/mol). No such sequence was found either downstream from the enzymatic-component genes or downstream from the binding-component genes in *C. perfringens* and *C. spiroforme* (Perelle et al. 1993, 1997; Gibert et al. 1997).

III. *C. Spiroforme* Toxin and CDT Proteins

A typical signal-peptide sequence precedes the coding regions for the mature proteins. The signal-peptide lengths of *C. spiroforme* toxin and CDT components ranges from 42 to 44 residues, which is usual for the Gram-positive bacteria (Figs. 1, 2).

The N-terminal protein sequencing of Sa indicates that the mature protein starts at Ser-47 (Perelle et al. 1993). However, Tyr-46–Ser-47 does not constitute a common cleavage site for bacterial signal peptidase. A preference-cleavage site (Ala–x) is found at Ala-44–Asn-45. Mature Sa could result from a signal-peptidase cleavage at Ala-44–Asn-45 and from a subsequent proteolytic removal of two N-terminal residues. Different putative cleavage sites are found in the equivalent position in Ia (Arg-41–Ala-42) and in CDTa (Lys-43–Val-44).

Trypsin-cleavage sites (Lys–Glu in Ib and CDTb, and Lys–Thr in Sb) are found immediately downstream from the predicted hydrophobic stretches ended by charged residues of the binding-component signal peptides. These cleavage motifs are presumably the signal-peptidase sites (Fig. 2).

The cleavage site (Ser-215–Gly-216) between the propeptide and mature Sb component has been determined by N-terminal protein sequencing. It is possible that Sb can result from cleavage at Ala-214–Ser-215 (which is a more

common proteolytic site) and subsequent removal of Ser-215. A trypsin-cleavage site (Lys-210–Leu-211) is found in the equivalent position in CDTb.

The predicted sizes and pI of the different components are similar, except for CDTa, which has an higher pI (9.3) than those of the other enzymatic components (Fig. 1). The alignments of the actin-ADP–ribosylating toxin sequences which have been characterized, as determined by the Clustal program, are shown in Figs. 4 and 5. The mature enzymatic and binding components, respectively, of the Iota-family toxins are very similar (83–85% identity), whereas the signal-peptide sequences are more divergent (40–61% identity). The functional domains have presumably been better conserved during evolution.

IV. C2-Toxin Genes and C2 Proteins

The C2 toxin genes characterized from *C. botulinum* C strain C203 (Fujii et al. 1996; Kimura et al. 1998) show the same organization as genes from the Iota family. The *c2I* Gene is 247 nucleotides upstream from the *c2II* gene, and both genes are transcribed in the same orientation.

The C2I and C2II proteins are shorter than the respective components of the Iota family. However, in contrast to the Iota proteins, no predicted signal-peptide sequences have been found in C2I and C2II. However, C2I is actually longer (10–15 amino acids) than the mature enzymatic components of the Iota family. The overall comparison of sequences shows that C2I is distantly related (29–32% identity) to the respective components of the Iota family whereas C2II shows an higher level of identity (40–42%). This is consistent with the lack of cross-immunoreaction between Iota and C2 components. The processing of C2II (721 amino acids) has not been determined. Two putative cleavage sites (Lys-179–Leu-180, Lys-181–Ala-182) lie in a position equivalent to those found in the binding components of the Iota family (Fig. 2). However, the predicted mature C2II (59.9kDa) has a lower MW than that experimentally determined (69kDa; Ohishi and Okada 1986; Kimura et al. 1998).

E. Relatedness of Actin ADP–Ribosylating Toxins with Other Toxins

I. Relatedness with ADP–Ribosylating Toxins

The enzymatic components of the Iota-toxin family show an overall identity of about 23.4% with the C3 enzymes (Chap. 11), which are also ADP–ribosyl transferases whose substrate is Rho. C3 enzymes are synthesized by *C. botulinum* C and D strains (which also produce C2 toxin), *C. limosum* and also by other non-clostridial bacteria, such as *Bacillus cereus* and *Staphylococcus aureus*.

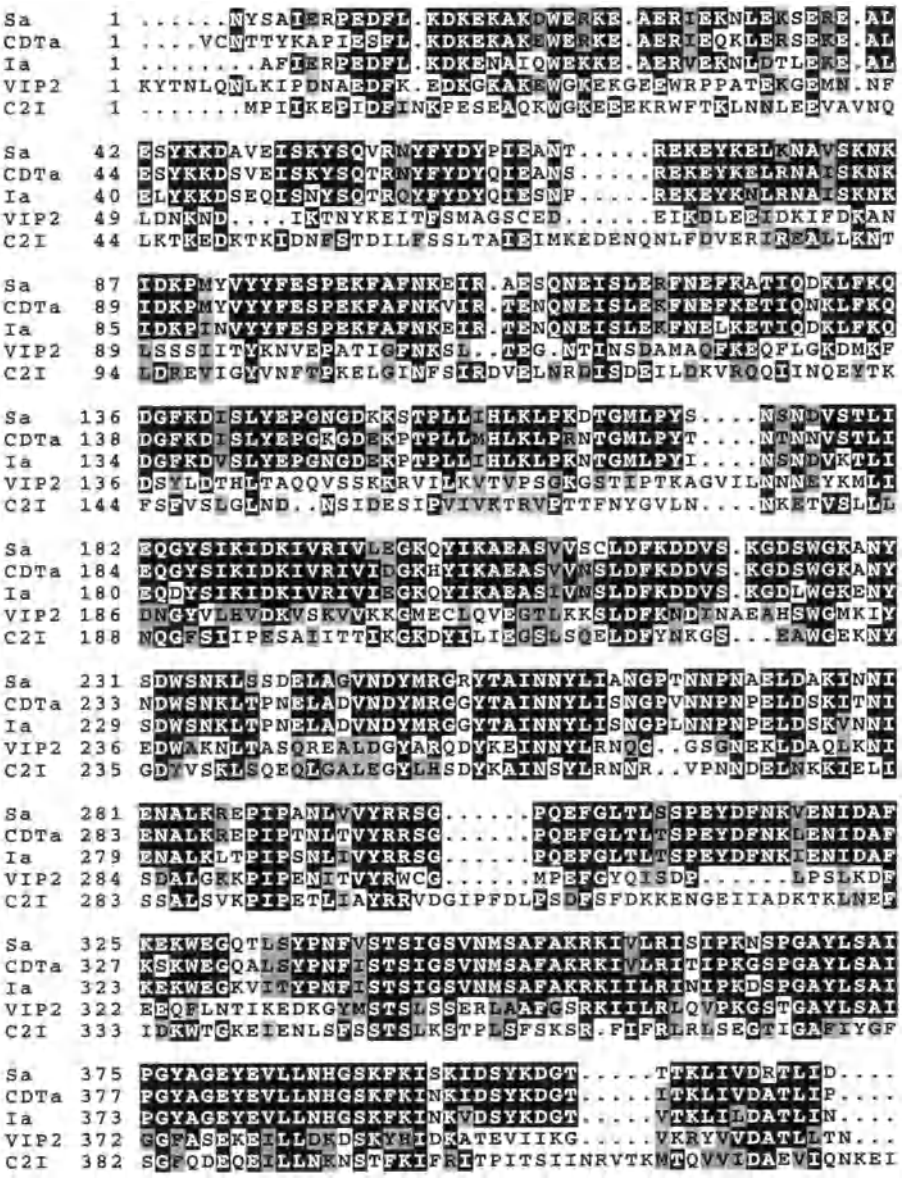

Fig. 4. Sequence alignment of the enzymatic components of the actin-adenosine diphosphate–ribosylating toxins by the Clustal program: Ia from *Clostridial perfringens* Iota toxin (Perelle et al. 1993); Sa from *C. spiroforme* toxin (Gibert et al. 1997); CDTa from *C. difficile* transferase (Perelle et al. 1997a); vegetative insecticidal protein 2 from *Bacillus thuringiensis* (Warren et al. 1996); and C2I from *C. botulinum* C2 toxin (Fujii et al. 1996). The signal peptides are not shown

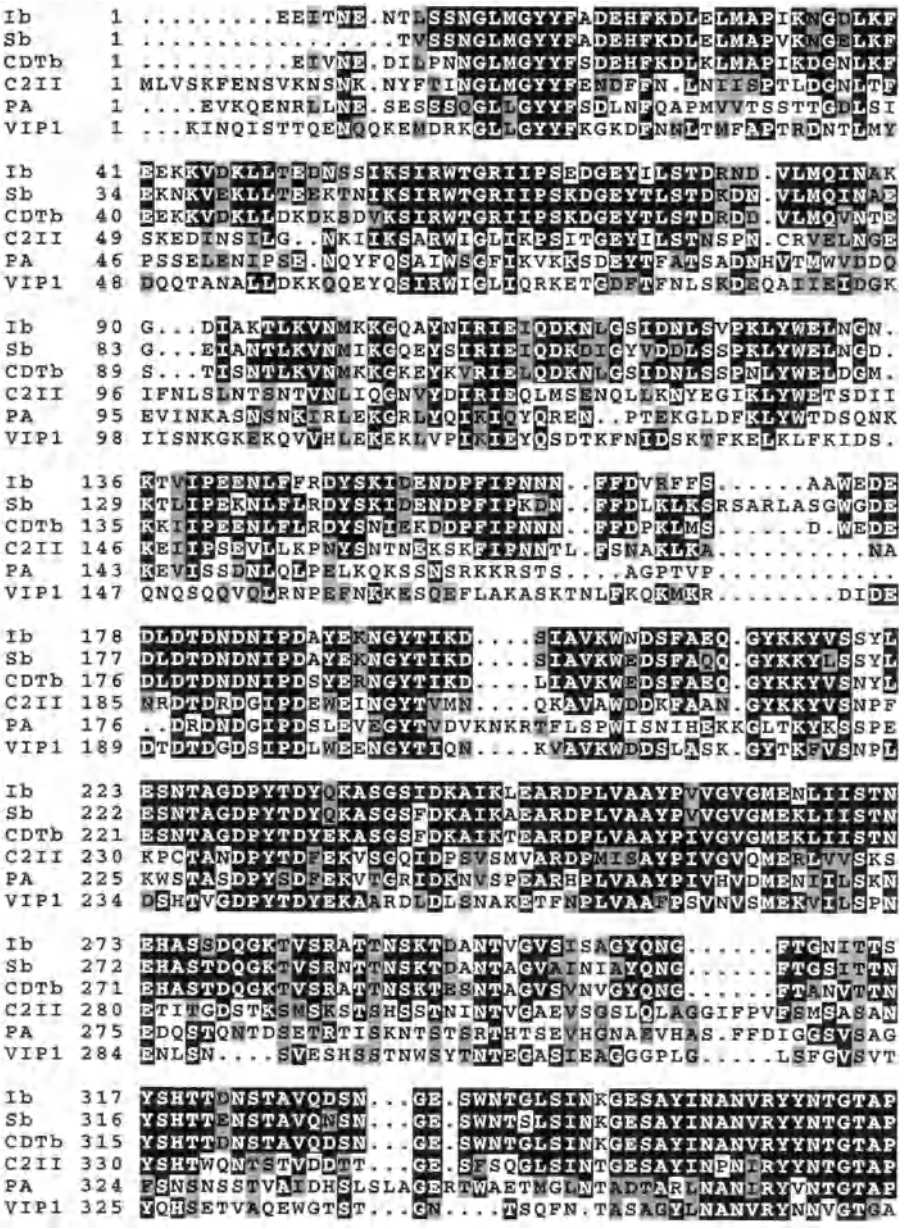

Fig. 5. Sequence alignment of the binding components of the actin-adenosine diphosphate–ribosylating toxins and of the antrax toxin (PA; protective antigen) by the Clustal program: Ib from *Clostridial perfringens* Iota toxin (PERELLE et al. 1993); Sb from *C. spiroforme* toxin (GIBERT et al. 1997); CDTb from *C. difficile* transferase (PERELLE et al. 1997a); C2II from *C. botulinum* C2 toxin (KIMURA et al. 1998); PA from *Bacillus anthracis* toxin (WELKOS et al. 1988); and vegetative insecticidal protein 1 from *B. thuringiensis* (WARREN et al. 1996). The signal peptides are not shown

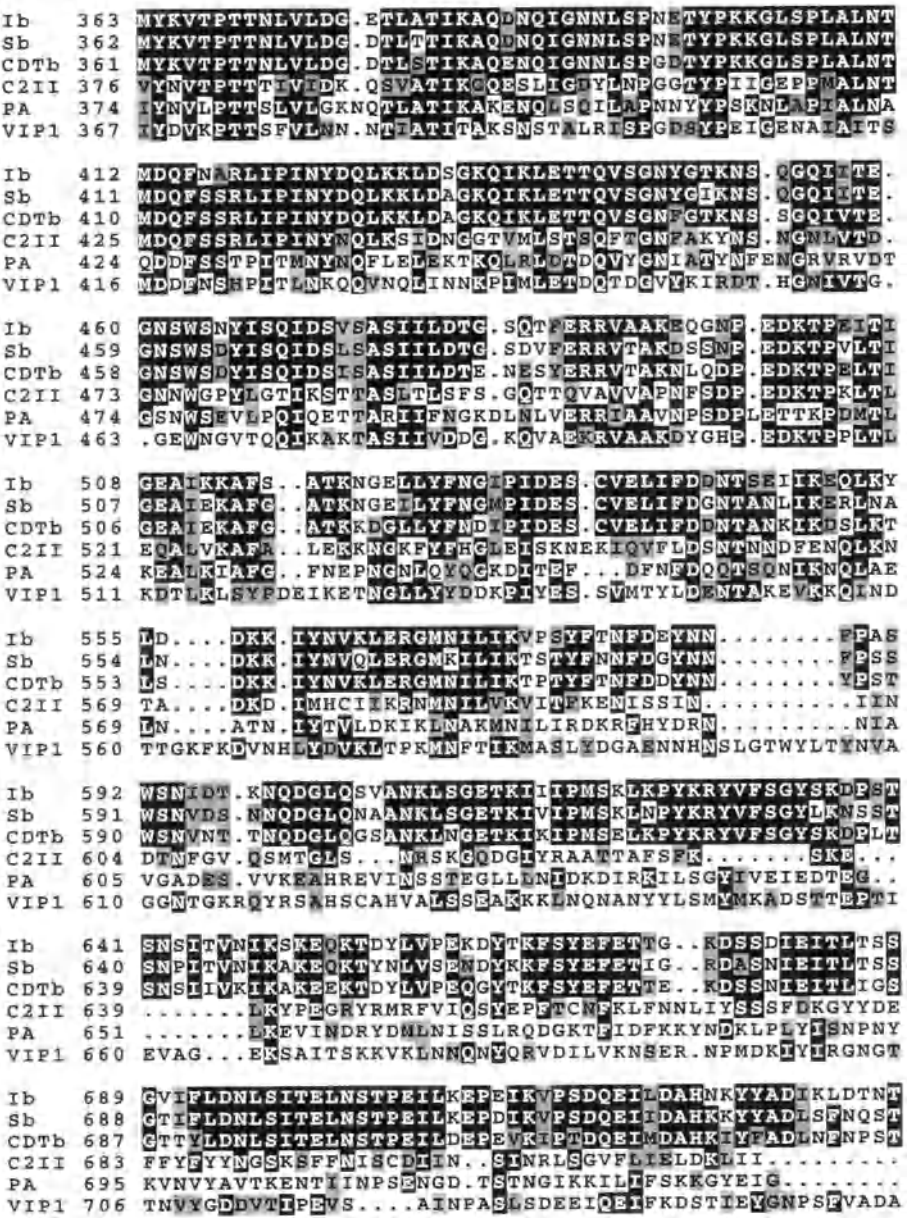

Fig. 5. *Continued*

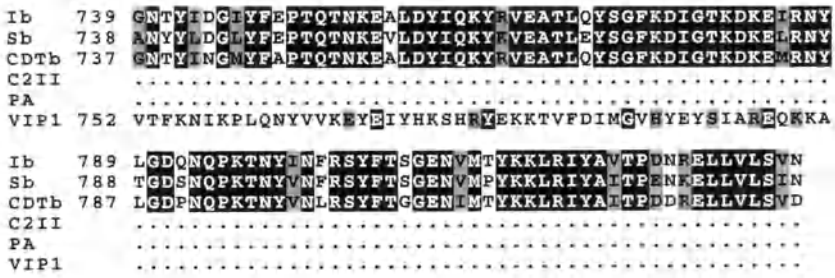

Fig. 5. *Continued*

Comparison of overall amino acid sequences shows no significant homology between actin-ADP–ribosylating toxins and the other ADP–ribosylating toxins. However, the ADP–ribosylation enzymatic site is conserved in actin ADP–ribosylating toxins, C3 enzymes and other ADP–ribosylating toxins (see below).

II. Relatedness with *Bacillus anthracis* Toxins

1. Sequence Homology

By comparison with protein databases, it has been found that Ib is significantly similar to the protective antigen (PA) of the anthrax toxins (33.9% identity; Perelle et al. 1993; Fig. 5). The anthrax toxins are also binary toxins consisting of three independent proteins. PA constitutes the binding component and can mediate the internalization into cells either of the edema factor (EF) or the lethal factor (LF), which are the enzymatic components active intracellularly (Chap. 22).

Sb and CDTb also show an overall identity of 38.4% and 37.6%, respectively, with PA, but C2II is less related (30.7% identity). The general structure of PA is conserved in the binding components of the actin-ADP–ribosylating toxins. These proteins are proteolytically split into three fragments: signal peptide, propeptide and active form. The 29 N-terminal residues of the PA sequence correspond to the signal peptide. The secreted PA (735 amino acids) has a MW of 82.7 kDa and is activated by a cellular protease (furin), which produces two fragments: one with a MW of 20 kDa (PA20) and one with a MW of 63 kDa. The longer fragment (PA63) is the active form. The secreted clostridial binding components are processed in a 20-kDa fragment and in an active form (74–80 kDa) which is slightly longer than PA63 (Fig. 2).

The PA crystal structure has recently been solved. PA consists of four domains. The 258 N-terminal residues correspond to domain 1, which consists of β-sheets, four small helices and a furin-cleavage site. The activation by furin releases the 167 N-terminal residues (PA20), and the residues 168–258 (domain 1') form a hydrophobic surface which is considered as the binding

site for EF and LF. Domain 2 (residues 259–487) contains a β-barrel core and a large, flexible loop implicated in membrane insertion. The function of the small domain 3 (residues 488–595) is unknown, and it is composed of six β-sheets and four helices. Domain 4 (residues 596–735) is slightly separate from domains 1, 2 and 3, which are closely associated. Domain 4, which contains a β-sandwich, is analogous to the antigen-binding loop of immunoglobulin and the receptor-binding loop of DT. This domain is involved in the recognition of the cell-surface receptor (Petosa et al. 1997). The corresponding amino acid sequences of Ib show 34, 41, 43 and less than 10% identity the domains 1, 2, 3 and 4 of PA, respectively, indicating that Ib probably shares the same folding of domains 1, 2 and 3. Similar results are obtained for Sb, CDTb, C2II and PA.

2. Immunological Relatedness

Ib and Sb share common epitopes with PA, but the level of cross-immunoreaction is low. Rabbit polyclonal antibodies raised against Sb or Ib cross-reacted with PA in immunoblot and, conversely, anti-PA antibodies recognized Ib and Sb. However, the level of cross-immunoblotting is low. A higher amount of PA than Sb (approximately 6000 times more) was required for a similar immunological reaction with anti-Sb antibodies and, conversely, approximately 250 times more Sb than PA was used with anti-PA antibodies (Perelle et al. 1997b).

ELISA titration, which is performed in non-denaturating conditions (in contrast to the immunoblotting technique), also showed important differences between the homologous and heterologous combinations. Anti-PA antibodies recognized high levels of PA, but failed to detect Ib and Sb (Table 2). Anti-Ib and anti-Sb antibodies reacted with Ib and Sb more effectively than with PA (approximately 1000 times more). Therefore, common epitopes coexist on the binding components of Iota toxins and *B. anthracis* toxins, but the overall level of immunological relatedness is low between the binding components of both toxin groups. Similar results were obtained between PA and C2-II. Anti-PA antibodies did not detect C2-II by ELISA, and anti-C2-II gave an ELISA titer 45 times higher with C2-II than with PA (Table 2).

3. Functional Comparison

No functional complementation was observed between Iota toxin and anthrax-toxin components. The binding components can form active toxins only in combination with their respective enzymatic components. Macrophages J774, which are sensitive to the lethal anthrax toxin (PA and LF), were also found to be target cells for the Iota toxin (Ib and Ia). This indicates that Ib is able to internalize Ia into macrophages J774. However, various combinations of PA with Ia induced neither morphological changes nor cytoskeleton modification. Identical results were obtained using CHO and Vero cells (Perelle et al. 1997b). Incubation of macrophages J774 with

LF and PA induces cell lysis, as assayed by the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide test. However, LF, even with large amounts of Ib, did not reduce macrophage viability. Thus, Ib is unable to mediate the translocation of EF or LF, and PA cannot internalize Ia (Perelle et al. 1997b).

The N-terminal regions of LF and EF (residues 1–250) are very similar and are involved in binding to PA (Leppla 1995). However, there is no significant homology between Ia and EF or LF, suggesting that the PA- and Ib-binding sites for the enzymatic components are different, and this supports the hypothesis that the binding components can interact only with their respective enzymatic components.

Other differences between Iota toxins and anthrax toxins reside in the recognition of the cell-surface receptor and in the mode of entry into cells. PA and Ib recognize different cell-surface receptors, since no competition has been evidenced between PA and Ib in combination with Ia, or conversely, between Ib and PA in association with LF, as assayed by the cytotoxicity of Iota and anthrax toxins, respectively (Perelle et al. 1997b). It is noteworthy that the C-terminal region of PA (domain 4, which is considered to be the receptor-binding domain; Petosa et al. 1997) and the equivalent region of Ib show a low level of identity (<10%). Anthrax toxins enter cells by receptor-mediated endocytosis, and translocation of the enzymatic component to the cytosol requires passage through an acidic compartment (Friedlander 1986). This entry pathway seems to be similar to that of C2 toxin but differs from that of Iota toxin, which is not routed by acidic vesicles, as mentioned above.

III. Relatedness with Other Binary Toxins

1. *Bacillus* Binary Toxins

Bacillus species produce many insecticidal toxins. The best known are the insecticidal crystal toxins, which are synthesized during sporulation (Porter et al. 1993; Porter 1996). *B. thuringiensis* and *B. cereus* also produce insecticidal proteins (VIPs or vegetative insecticidal protein) during the vegetative growth phase. VIPs are binary toxins and consist of VIP2 (462 amino acids) and VIP1 (833 amino acids). The proteins are synthesized with a signal peptide (49 N-terminal residues of VIP2 and 33 N-terminal residues of VIP1) and are secreted into the culture supernatant. In addition, the 184 N-terminal residues (propeptide) of the secreted VIP1 are excised, and the mature form is a 80-kDa protein (Warren et al. 1996). VIP1 corresponds to the binding component and shows a high sequence similarity with PA over the first 600 residues. Therefore, the folds for domains 1, 2 and 3 of PA seem to be conserved in VIP1 (Petosa et al. 1997). The sequence identity between VIP1 and the binding components of clostridial binary toxins reaches 30–31%.

The enzymatic activity of VIP2 has not yet been reported. This component shows 29–31% identity with the enzymatic components of the clostridial binary toxins. Moreover, the residues involved in the catalytic site for ADP–ribosylation (see below) are conserved in VIP2, and this protein displays an affinity for nicotinamide adenine dinucleotide (NAD; WARREN et al. 1996).

VIP2 from *B. thuringiensis* and *B. cereus* are closely related (91% identity), and VIP1s from both bacterial species show an overall 77% identity. However, the similarity is not homogeneous along the proteins. Two thirds of the N-terminal parts are highly related (91% identity), whereas one-third of the C-terminal parts is less homologous (35% identity). The difference between the putative receptor-binding domains could reflect the different patterns of sensitive insects, which are the targets of both groups of toxins. VIP1 from *B. thuringiensis* and *B. cereus* can be interchanged to form fully active toxins in combination with VIP2 (WARREN et al. 1996). Binary toxins from *B. thuringiensis* and *B. cereus* can be considered as a distinct family distantly related to the clostridial actin-ADP–ribosylating toxins.

B. sphaericus strains also produce an insecticidal binary toxin. This toxin consists of 51-kDa and 42-kDa components which are not significantly related to the clostridial binary toxins. The 51-kDa protein is considered as the cell-binding component. Both components are internalized into cells, leading to cell lysis by an unknown mechanism (PORTER et al. 1993).

2. Leukocidins and γ-Lysins

Leukocidins produced by *S. aureus* are binary toxins which are cytotoxic to polymorphonuclear leukocytes. These toxins consist of two proteins (S and F) which are, individually, not cytotoxic. Both components are required in order to induce cytotoxic changes. The major effect is observed at an equimolar ratio of the S and F components.

The genes encoding the S (315 residues) and F (323 residues) components are separated by only one nucleotide. A typical signal peptide is present at the N-terminal part. The mature proteins have a size of 31 kDa (S) and 32 kDa (F). S binds to GM1 ganglioside on cell membranes of sensitive leukocytes and catalyzes the ADP–ribosylation of a 37-kDa membrane protein. This protein induces phospholipid methylation and phospholipase-A2 activation and increases the number of binding sites for the F component. The F component, bound to the cell membrane in the presence of S component, ADP–ribosylates a 41-kDa membrane protein, which subsequently stimulates a phospholipid inositol-specific phospholipase C. This results in disorganization of the cell membrane, and cell lysis (NODA 1995).

γ-Lysins are also binary toxins that share strong sequence homology with leukocidins but use a different mode of interaction with cell membranes (FERRERAS and MENESTRINA 1997).

No significant sequence identity is observed between clostridial binary toxins, leukocidins and γ-lysins.

F. Genetics of the Actin-ADP–Ribosylating Toxins

I. Genomic Localization

The genomic localization of Iota-toxin genes has been mapped using DNA–DNA hybridization with CsCl-purified plasmids, whole DNA and specific probes. In four *C. perfringens* strains tested, *iap* and *ibp* genes were localized on a large plasmid (120–140 kbp; Gibert et al. 1997). Using pulse-field gel electrophoresis, *iap* and *ibp* in an additional strain have also been found to be plasmid borne (Katayama et al. 1996). Thus, the genes of the major toxins, including Beta, Epsilon and Iota toxins, which contribute to the toxinotyping of *C. perfringens*, are present on extrachromosomal elements. The gene of *C. perfringens* enterotoxin (*cpe*), which is responsible for food intoxication symptoms, is either located on a variable region of the chromosome (mainly in strains involved in human food poisoning) or on a plasmid mainly in non-food-borne human gastrointestinal diseases and veterinary isolates (Cornillot et al. 1995; Collie and McClane 1998). In two of four *C. perfringens* E strains, the *cpe* gene is localized on the same plasmid that contains the Iota-toxin genes. However, CPE production has not been evidenced in these strains. The presence of a silent *cpe* gene, indicated by the presence of nonsense and frameshift mutations, has also been reported in other *C. perfringens* E strains (Bellington et al. 1998; Wieckowski et al. 1998).

Sequence variation is commonly observed in Iota-toxin genes. Among the four *C. perfringens* E strains tested, three different restriction-hybridization patterns were observed using *iap* and *ibp* probes. In addition, partial sequencing of the *iap* gene from two different strains showed a 20% nucleotide difference. The intraspecies Iota-toxin-gene variation is apparently the same as that observed among *C. perfringens*, *C. spiroforme* and *C. difficile* strains (Gibert et al. 1997).

One *C. perfringens* E strain contains only the *iap* gene. The lack of *ibp* gene was also confirmed by the absence of production of Ib. Culture supernatants did not induce an Iota-cytotoxic effect on Vero cells, but it was able to ADP–ribosylate cellular actin in vitro (Gibert et al. 1997). A similar feature was observed in two *C. difficile* strains, which only contain the gene coding for the enzymatic component (Gibert et al. 1997).

In *C. spiroforme* and *C. difficile*, the ADP–ribosylating-toxin genes have been localized on the chromosomal DNA, even those strains contain large plasmids similar in size to the *C. perfringens* plasmids (Fig. 6). Two different *C. spiroforme* strains showed an identical hybridization pattern, suggesting that these strains have similar coding sequences for the toxin genes and a similar chromosomal localization. A hybridization pattern different from that of *C. spiroforme* was observed in *C. difficile* (Gibert et al. 1997).

C2-toxin gene is located on the chromosome in *C. botulinum* C and D. In contrast, the botulinum neurotoxin C1 and D genes and the C3 gene have a different localization and are on phage DNA. The strains, which have lost their

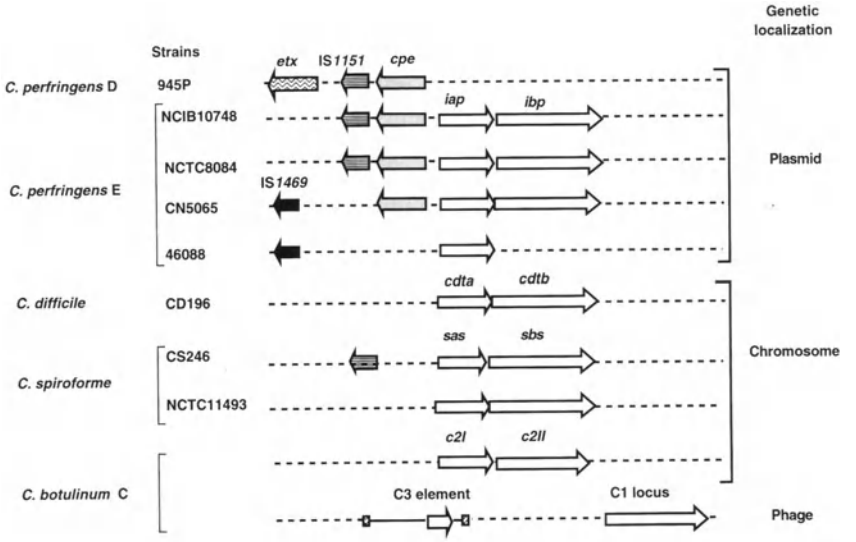

Fig. 6. Genomic localization of actin-adenosine diphosphate–ribosylating toxin genes in *Clostridium perfringens*, *C. difficile* and *C. spiroforme* (Gibert et al. 1997). *cpe*, *C. perfringens* enterotoxin gene, *etx*, epsilon toxin gene

toxigenic phages, only produce the C2 toxin (Rubin et al. 1988), and a C2-gene probe hybridized with genomic DNA but not with phage DNA (unpublished). Similar results were obtained by PCR. Specific DNA amplification of the C2 gene was only achieved with genomic DNA from *C. botulinum* C and D and was unsuccessful with phage DNA (Fujii et al. 1996).

II. Gene Transfer

The sequence homology between the clostridial binary toxins and their conserved domain organization suggest that their genes are derived from a common ancestor. The percentage of guanosine and cytosine (GC%) in the coding region (26–28%) is similar to the GC% in the clostridial genomes, indicating that the ancestor gene is a clostridial gene or is from an organism genetically related to Clostridia. The relatedness between the binding components of clostridial binary toxins, PA and VIP1 from *Bacillus* may support a common origin for these genes.

According to the degree of similarity, divergence of the C2-toxin genes from the Iota-toxin genes may have occurred earlier than the separation of the members of the Iota-toxin family. The high homology level between the toxins of the Iota family strongly supports an inter- and intraspecies gene transfer. In *C. perfringens* E, the Iota-toxin genes, which are located on plasmids, may have been transferred between *C. perfringens* strains by conjugation and mobilization of large plasmids. Conjugation and mobilization of large

plasmids in *C. perfringens* have already been reported (Brefort et al. 1977). This mechanism is probably involved in two *C. perfringens* E strains that contain identical large plasmids harboring the *iap*, *ibp*, *cpe* and the insertion sequence *IS1151* and share an identical enzymatic restriction pattern (Fig. 6; Gibert et al. 1997). Toxinotype changes that have been observed in some *C. perfringens* strains may result from loss or acquisition of toxigenic plasmids (Katayama et al. 1996; Yamagishi et al. 1997). However, two other *C. perfringens* E strains that have been analyzed, contain different plasmids and, in *C. spiroforme* and *C. difficile*, the Iota-toxin-related genes are located on the chromosome. Gene mobilization between plasmids and chromosomes is generally mediated by insertion sequences or transposons. Insertion sequences, such as *IS1151* and *IS1469*, have been identified in the flanking regions of *cpe* and the Epsilon-toxin genes (Daube et al. 1993; Brynestad et al. 1997). These elements have not been directly associated with the Iota-toxin genes in *C. perfringens* E (Fig. 6), and *IS1151* was identified in only one strain of *C. spiroforme*.

Mobile elements, which could mediate mobilization of the Iota-toxin genes, have not been identified. The DNA regions containing the Iota-toxin genes show that the coding sequences are highly related (80% identity), whereas the promoter and 3' non-coding regions are less related (44% and 60% identity, respectively). Multiple rearrangements and partial deletion of the mobile elements could have been be involved subsequently to the toxin-gene transfer. The finding that a *C. perfringens* strain and several *C. difficile* strains only contain the enzymatic-component gene indicates that enzymatic- and binding-component genes can evolve separately.

G. Gene Expression

I. Genes of Enzymatic and Binding Components are Organized in an Operon

In all the Clostridial strains that have been studied, the actin-ADP–ribosylating-toxin genes show the same organization: (1) the two genes encoding the enzymatic and binding components are in close vicinity, (2) the enzymatic-component gene is usptream from the binding-component gene, and (3) both genes are transcribed in the same orientation. Northern analysis has been performed in *C. difficile* (Perelle et al. 1997a) and in *C. perfringens* E (manuscript in preparation). Only long messenger RNA (mRNA) covering both genes has been evidenced. This indicates that the *Clostridium* binary-toxin genes are organized in an operon.

Transcriptional analysis has been performed in *C. perfringens* E NCIB10748. Two transcriptional start sites (S1 and S2) have been mapped upstream of the initial codons of *iap*, and one (S3) upstream of the initial codon of *ibp*. S1 seems to be the major transcriptional start site, since the

labeling was stronger than that of S2 in a primer-extension experiment. The promoter deduced from S1 shows conserved –10 (TATAAT) and moderate homologous –35 (GAGATT) regions in the *Clostridium* consensus sequences for these elements (YOUNG et al. 1989). However, the promoter regions deduced from S2 and S3 are weakly related to the consensus sequences (manuscript in preparation).

II. Gene Regulation

The *iap*-gene promoter region displays a particular feature consisting of an inverted repeat between the –10 box and the initial codon of *iap* able to form a stable stem and loop structure. The transcriptional start site, S1, is positioned at the 5′ extremity of the inverted repeat. Moreover, three repeat motifs similar to the DNA-binding site of Hpr, which is a transition-state regulator in *Bacillus subtilis*, are localized in the –10 promoter region corresponding to S1 and in the stem and loop structure. However, Iota-toxin production is not dependent on sporulation and occurs during the exponential-growth phase.

Various constructions have been performed to analyze the expression of the Iota-toxin genes (Fig. 7). *iap* And *ibp* were expressed separately with their own promoter regions, but at lower levels than those obtained with the whole operon. *ibp*, under the control of the *iap* promoter, was only expressed at a low level (manuscript in preparation). This indicates that each gene can be expressed separately, but the integrity of the whole operon is required for the

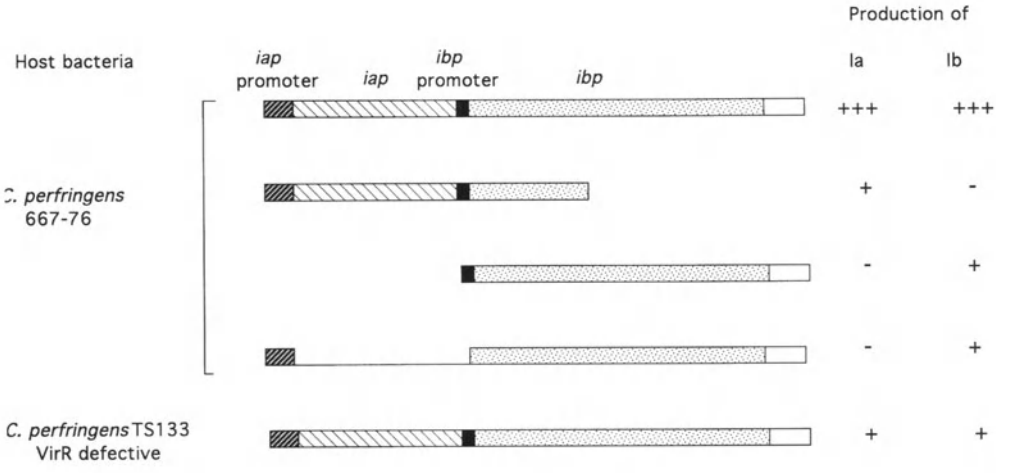

Fig. 7. DNA constructions of *iap* and *ibp* genes (with various promoter regions in the vector pJIR750), production of Iota-toxin components Ia and Ib in *Clostridium perfringens* assayed by Western blotting, and cytotoxic activity of the culture supernatants in Vero cells

optimum level of Ia and Ib production. In the wild-type strain, bicistronic expression is predominant, since mRNA corresponding to individual genes was not detected.

The deletion of the stem and loop structures, even associated with mutation of the Hpr motif in the –10 sequence of the *iap* promoter, did not alter significantly the expression level of *iap* and *ibp* (Fig. 7). Therefore, the hairpin structure of the *iap* promoter region does not seem to be directly involved in the regulation of the Iota-toxin genes.

A bicomponent system termed VirR–VirS has been found to regulate the production of different *C. perfringens* toxins, such as Alpha toxin, Theta toxin (or perfringolysin) and collagenase, whose genes are localized on the chromosome (ROOD 1998). We have tested whether Iota toxin, which is plasmid encoded, is also regulated by VirR–VirS. The recombinant plasmid containing the whole Iota-toxin operon was transferred into a *C. perfringens* strain defective for the VirR gene (SHIMIZU et al. 1994). The cytotoxic activity of the culture supernatant was 128 times lower than that in the parental strain, S13. However, the production of Ia and Ib, assayed by Western blotting, was the same in both strains. Ib was unprocessed in the VirR-negative strain, and a complete activation was obtained by addition of α-chymotrypsin. This indicates that the protease that activates Ib is regulated by the VirR–VirS system in strain S13 (manuscript in preparation).

The *iap* promoter is not conserved in *C. difficile* and *C. spiroforme*. An inverted repeat lies in the region promoter of *cdtA*. However, no repeats or consensus binding sites have been found in the region promoter of the enzymatic gene in *C. difficile* and *C. spiroforme*.

In *C. difficile*, the production of CDTa and CDTb is about 40 times lower than that of Iota toxin in *C. perfringens*. This coincides with a lower amount of *cdtA* and *cdtB* mRNAs in *C. difficile* than of Iota-toxin genes in wild-type *C. perfringens*. A low level of *cdtA* and *cdtB* gene transcription or an unstability of the transcripts could be involved (PERELLE et al. 1997a). The expression of *cdtA* and *cdtB* genes seems to be equivalent to the basal expression of Iota-toxin genes in *C. perfringens*, and a regulation system able to increase the expression of these genes is probably lacking in the tested *C. difficile* strain. However, it is possible that CDT production in the digestive tract is higher than that in vitro.

In *C. botulinum* C and D, C2 toxin is produced during sporulation. C2 toxin is detected in the culture supernatant by the mouse lethal test when the spore population is higher than 10^4/ml (NAKAMURA et al. 1978). The regulation mechanism is not yet known. Another example of a Clostridium toxin that is dependent of sporulation is the *C. perfringens* enterotoxin (CPE). Three consensus DNA-binding sequences for the *B. subtilis* transition-state regulator Hpr have been identified upstream and one downstream of the coding sequence of the *cpe* gene (BRYNESTAD et al. 1994). Moreover, three promoter sites similar to SigK and SigE, which are sporulation-associated σ factors in *B. subtilis*, have been evidenced in the *cpe* promoter region (ZHAO and

MELVILLE 1998). No such feature has been found in the upstream region of the C2-toxin gene.

H. Identification of Actin-ADP–Ribosylating-Toxin-Producing Clostridia by Genetic Method

Typing of *C. perfringens* and other toxigenic Clostridia is classically performed by the mouse lethal test and seroprotection with specific anti-serum. Several PCR- and DNA-hybridization-based detection tests have been developed. Individual PCR for each toxin gene or multiplex PCR consisting of a primer mix specific to two or three toxin genes (including Iota-toxin gene) has been proposed for *C. perfringens* typing (SAITO et al. 1992; DAUBE et al. 1996; MOLLER and AHRENS 1996; SONGER and MEER 1996; FACH and POPOFF 1997; YAMAGISHI et al. 1997). The genetic method is a reliable and more rapid method than the classical test and avoids handling of laboratory animals. It can be used directly with clinical samples for detection of toxigenic Clostridia without enrichment culture if the *Clostridium* population in the sample is at least 10^5/ml (FACH and POPOFF 1997). This method is of particular interest for the detection of the *C. botulinum* C2-toxin gene, the expression of which is dependent on sporulation and requires special growth conditions.

I. Functional Domains

1. Enzymatic-Component Domains

a. Enzymatic Site

The enzymatic component is able to catalyze the ADP–ribosylation of G-actin but it cannot enter the cells in the absence of the binding component. The enzymatic site on the C-terminal part of Ia has been characterized. A truncated protein lacking the 208 C-terminal residues was inactive (PERELLE et al. 1996). By using affinity labeling with ultraviolet light in the presence of radioactive NAD, it was determined that the peptide Gly-363–Lys-388 binds to NAD and that Glu-378 plays a critical role in the active site (VAN DAMME et al. 1996).

Sequence analysis shows that the residues forming the ADP–ribosylation active site in all the ADP–ribosylating toxins are conserved in the actin ADP–ribosylating toxins. The active site consists of a NAD-binding cavity constituted of a β-strand and an α-helix flanked by two residues important for the catalytic activity (ARG or HIS, and GLU; DOMENIGHINI and RAPPUOLI 1996). The actin-ADP–ribosylating toxins are less related to the DT group than to the CT group, which comprises toxins active on heterotrimeric G-proteins [CT, LT, pertussis toxin (PT)] and on small G-proteins (C3 enzymes; DOMENIGHINI and RAPPUOLI 1996; Fig. 8). In the CT group, the active site has

```
                                    ββββαααααααααααα
Ia      YRR.296...(39).....FISTSIGSVNMSAFAKRKI.354....(23)....EYE.380
Sa      YRR.298...(39).....FVSTSIGSVNMSAFAKRKI.356....(23)....EYE.382
CDTa    YRR.300...(39).....FISTSIGSVNMSAFAKRKI.358....(23)....EYE.384
C2I     YRR.300...(44).....FSSTSLKSTOLS.FSKSRF.363....(23)....EQE.389
VIP2    YRW.301...(34).....YMSTSLSSERLAAFGSRKI.353....(23)....EKE.377
C3      FRG.90....(43).....YISTSL..MNVSQFAGRPI.148....(23)....QLE.174
PT      YRY.10....(39).....FVSTSSSRRYTEVYLEHRM.57....(72)....QSE.130
CT      YRA.8.....(52).....YVSTSTSLRSAHL.VGQTI.75.....(33)....EQE.111
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Fig. 8. Alignment of the residues forming the enzymatic site of the *Clostridium perfringens* Iota toxin (Ia; PERELLE et al. 1993), *C. spiroforme* toxin (Sa; GIBERT et al. 1997), *C. difficile* transferase (CDTa; PERELLE et al. 1997a), *C. botulinum* C2 toxin (C2I; FUJII et al. 1996), *C. botulinum* C3 enzyme (C3; POPOFF et al. 1991), pertussis toxin (PT; DOMENIGHINI and RAPPUOLI 1996) and cholera toxin (CT; DOMENIGHINI and RAPPUOLI 1996). Numbers in *brackets* correspond to nucleotide spacing

an arom-ph-Ser-Thr-Ser-ph consensus (arom represents an aromatic residue and ph a hydrophobic residue) which is preceded by an Arg catalytic residue instead of His, as in the DT group (DOMENIGHINI and RAPPUOLI 1996). A predicted β-strand and α-helix structure lies in equivalent positions in Ia (337–354), Sa, CDTa and C2I (Fig. 8). The residues [STS(I/L)] forming the β-strand are highly conserved in the enzymatic components and in C3 enzymes, CT, LT and PT, whereas those involved in the α-helix are less conserved. The residues (Arg and Glu) flanking the NAD cavity, which have been found to be essential for catalysis in PT, CT and LT, match Arg-295 and Glu-380 of Ia and are conserved in all the enzymatic components and C3 enzymes. In Ia, substitution (by site-directed mutagenesis) of Arg-295 by Lys, and Glu-380 by Asp or Ala induced a pronounced decrease of the in vitro ADP–ribosylation activity with actin as substrate (Table 4). The mutated proteins, when introduced into Vero cells in the presence of Ib, were not cytotoxic. Glu-378, which has been found to cross-link NAD (VAN DAMME et al. 1996), has been replaced by Ala. The resultant protein was inactive in vitro and in vivo. The residue immediately downstream of Arg-295 is also an Arg in Ia, Sa, CDTa and C2I. The Arg-296–Lys substitution caused only a slight diminution of activity. This shows that Arg-295, Glu-378 and Glu-380 are critical residues for the ADP–ribosylation activity of Ia and the actin depolymerization effect in cells. This confirms that the intracellular ADP–ribosylation of actin by Ia is responsible for the cytotoxic effects of Iota toxin. Recently, identical results have been found in C2I (BARTH et al. 1998b).

The biglutamic motif (Glu-378–x–Glu-380) of Ia is conserved in equivalent positions in Sa, CDTa, C2I, VIP from *B. cereus* and *B. thuringiensis*, mosquitocidal toxin from *B. sphaericus*, CT, LT and mammalian ADP–ribosyltransferases (DOMENIGHINI and RAPPUOLI 1996; PERELLE et al. 1996; VAN DAMME et al. 1996; WARREN et al. 1996). This structure probably participates in NAD binding and in the catalytic reaction. In PT, C3 from *C.*

Table 4. Effect of amino acid substitution on Ia activity. The activities of Ia mutants tested by in vitro adenosine triphosphate–ribosylation and cytotoxicity in Vero cells in the presence of the Ib component were unchanged (–) or dramatically decreased (↓) compared with that of wild type Ia

Amino acid substitution	Activity compared with that of wild-type Ia
Glu-14–Ala	–
Trp-19–Ser	–
Arg-295–Lys	↓
Arg-296–Lys	–
Glu-378–Ala	↓
Glu-380–Asp	↓
Glu-380–Ala	↓

botulinum, *C. limosum*, *B. cereus* and *S. aureus*, the first Glu is replaced by a Gln. The enzymatic site is localized in the C-terminal part of the actin- and Rho-ADP–ribosylating transferases, whereas it lies in the N-terminal part of toxins, such as DT, CT, LT and PT, which recognize elongation factor 2 or heterotrimeric G-proteins.

2. Enzymatic-Component Domain which Interacts with the Binding Component

The domain of the enzymatic component which interacts with the binding component during the cell-intoxication process has been investigated in Iota toxin (Marvaud and Popoff, submitted). Various fragments of Ia generated from the inactive mutant Glu-380–Ala were fused to the C-terminal part of C3 enzyme (Fig. 9). All the fusion proteins retained the C3-enzymatic activity, ADP–ribosylated Rho and were inactive on actin. In a competition assay on Hep2 cells, the fusion proteins containing the central domain (residues 87–288) were the most able to compete with wild-type Ia to bind to Ib. Internalization of the chimeric molecules into cells in the presence of Ib was monitored by actin-cystoskeleton disruption during immunofluorescence and by *in vivo* modification assay of Rho. The construction encompassing the central and C-terminal parts of Ia was the most effective. This indicates that the Ia central domain plays a major role in the binding to Ib and that the central and C-terminal parts are required for efficient translocation into the cells. The C-terminal part could induce or stabilize an optimal conformation of the molecule for the translocation.

The N-terminal part of C2I fused to the C3 enzyme is able to drive the recombinant molecule into cells. The functional domains in C2I have not yet been clarified (Barth et al. 1998).

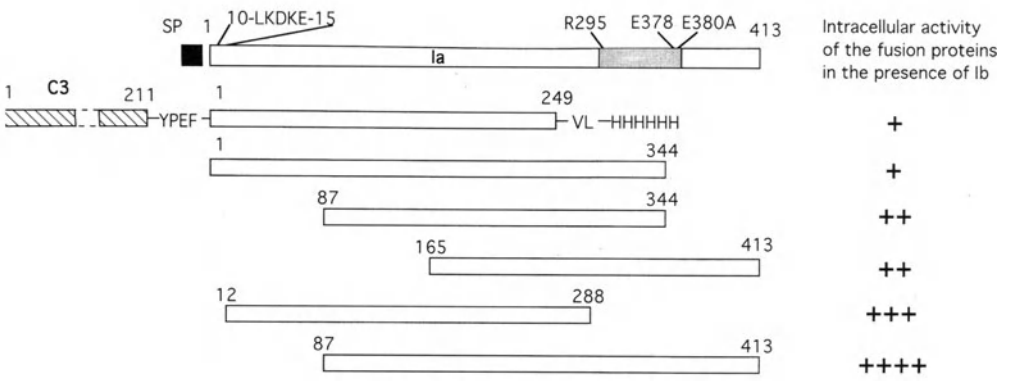

Fig. 9. Fusion proteins between the C3 enzyme and various fragments of mutated Ia (E380A). In Vero cells, the activity of the recombinant toxins in the presence of Ib was determined by assay of the actin-cytoskeleton depolymerization

3. Actin-Binding Site

A motif (LKDKE) involved in the actin-binding sites of several actin-binding proteins (Prekeris et al. 1996) is conserved in the N-terminal part of Ia (positions 10–15), Sa (positions 9–14) and CDTa (positions 14–19). In C2I, this motif is slightly different (44-LKTKE-48) and could reflect the difference in the substrate recognition. C2 toxin ADP–ribosylates non-muscular actin, whereas Iota toxin is active on all the actin isoforms. Experimental evidence that this motif is involved in the actin binding of the Iota and C2 toxins remains to be demonstrated.

II. Binding-Component Domains

The binding components are secreted in an inactive form which is activated by proteolytic removal of a 20-kDa N-terminal peptide (Fig. 2). The activation of the Iota-toxin-binding components is not accompanied by a significant change of the isoelectric point (Fig. 2), contrary to the case for Epsilon toxin, which is also produced by *C. perfringens*. The active form of Epsilon toxin (28.6 kDa, pI 5.36) derives from the protoxin (32.3 kDa, pI 8.02) by the excision of 13 basic N-terminal and 29 C-terminal residues (Minami et al. 1997). Iota toxin is usually activated by endogenous proteases during the growth phase of *C. perfringens*. Therefore, trypsinization of the culture supernatant does not increase the biological activity of the Iota toxin (Stiles and Wilkins 1986a).

In *C. botulinum* C and D, C2II is not activated by bacterial proteases and can be activated by trypsin or chymotrypsin. Under normal conditions, digestive proteases can activate C2 toxin. The activation of C2II is accompanied by a change in the MW from 101 kDa to 88 kDa (Ohishi 1987). The trypsinized and untrypsinized forms of C2II are able to bind to cell surfaces and brush borders of mouse intestine. However, only activated C2II induces the subse-

quent binding of C2I and elicits the biological activity (Ohishi and Miyake 1985). C2II also has hemagglutinating and hemolytic activities. Activated C2II shows a stronger hemagglutinating activity than untrypsinized C2II in human and animal erythrocytes. This suggests that activation possibly results in a conformational change of the binding-receptor domain, which is more able to interact with the erythrocyte-binding sites (Sugii and Kozaki 1990).

CDTb is partially cleaved by *C. difficile* proteases. However, CDTb is inactive in the culture supernatant, and trypsinisation is required to convert CDTb in its active form (Perelle et al. 1997a). Likewise, *C. spiroforme* cultures contain the non-active form of the toxin, and trypsinization is needed to obtain biologically active toxin (Fernie et al. 1984).

The sequence homology of the PA domains 1′ and 2 (which are involved in binding to the enzymatic component and to the translocation function, respectively) with the corresponding regions of Ib, Sb, CDTb and C2II supports the hypothesis that same domain organization exists in the binding components of actin-ADP–ribosylating toxins (Petosa et al. 1997). Therefore, the N-terminal parts of the binding components are probably involved in interaction with the enzymatic component and in the translocation function, and the C-terminal parts presumably correspond to the receptor-binding domains. This domain organization is also supported by the presence of a predicted transmembrane segment in the N-terminal part of Ib (positions 253–269), Sb, CDTb and C2II, which is equivalent to domain 2 of PA. Transmembrane segments are generally associated with the translocation domains of various toxins, such as DT (Choe et al. 1992). Substitutions of the hydrophobic residues (Ala-255 and Pro-258) in the predicted transmembrane region of Ib by a charged residue (Glu) yielded unstable recombinant proteins possibly by changing the conformation, thereby facilitating proteolytic cleavage at sites that would otherwise be concealed. This suggests that this region, at least, is critical for the stability of the molecule (Gibert et al. 1997).

A consensus ATP/GTP-binding motif (ASxxQGKT) is localized downstream from the hydrophobic region in Ib, Sb and CDTb. This motif seems to be non-functional, or at least non-essential for the Ib activity, since the Ib mutant (Lys-281–Ala) is as active as the native Ib form (Gibert et al. 1997).

The active form of C2II tends to form oligomers of MW 365 kDa, which could correspond to tetra- or pentamers, whereas the untrypsinized C2II was only found as a monomer. The lethal activity of the oligomer and monomer forms combined with C2I was approximately the same. This suggests that C2II acts in vivo as a monomer, but it is possible that C2II forms oligomers on the cell surface (Sugii and Kozaki 1990). Moreover, C2II oligomers have hemagglutinating and hemolytic activities, whereas the monomer only has hemagglutinating activity. It has been reported that C2II is able to form channels across lipid-bilayer membranes. The chemical structure of the C2 channel is not known; it could result from C2II oligomerization (Schmid et al. 1994). PA from anthrax toxin associates in heptamers at acidic pH. Despite the sequence homology with PA, Ib and Sb do not form oligomers, or do so only in small

proportions, under the same conditions (Perelle et al. 1997b). However, it is possible that the oligomerization of Iota-toxin-binding components requires specific conditions or only occurs when associated with cell membranes.

K. Concluding Remarks

The actin ADP–ribosylating toxins induce pathological disorders by interacting specifically with the actin cytoskeleton. For this reason, they constitute invaluable tools for study of the involvement of the actin cytoskeleton in various cell functions. A peculiarity of these toxins is the fact that they are binary toxins, constituted of two independent protein chains. We can suppose that this structure permits a more efficient entry of the toxin into cells or a more efficient activity of the enzymatic component inside the cell than is permitted by a one-chain toxin. The binary structure is advantageous in designing transporter proteins to deliver heterologous proteins into cells. Such models have been developed with C3 enzyme and C2 or Iota toxins (Barth et al. 1998a; Marvaud and Popoff 1998). Chimeric toxins are valuable as tools in cell biology and as therapeutic agents. The binding and enzymatic components of the binary toxins can be inoculated separately, and thereby the cell specificity can be increased and the effects of the toxin can be modulated (Simpson 1997).

The actin ADP–ribosylating toxins are mostly produced by Clostridia. They seem to have evolved from a common ancestor which was transferred and which has accumulated sequence variations in various *Clostridium* species. Two families (Iota and C2) which differ in their immunological properties and mode of entry into cells can be recognized. The selection pressure of this diversification and the advantage for a bacterium that produces these toxins remain to determine. The toxin variants are used to compare these toxins and to determine and study their functional domains.

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CHAPTER 14

Molecular Mechanisms of Action of the Large Clostridial Cytotoxins

I. JUST, F. HOFMANN, and K. AKTORIES

A. Introduction

The family of large clostridial cytotoxins consists of toxin A and toxin B from *Clostridium difficile*, the lethal toxin and the haemorrhagic toxin from *C. sordellii*, and the α toxin from *C. novyi* (Table 1). The comparable cytotoxic activities and the similar structures of the toxin molecules have led researchers to group them as one family (BETTE et al. 1991). This former phenomenological classification has turned out to be correct because of their almost identical enzymatic ability to modify comparable target proteins. The in vivo effects of the toxins, however, differ from each other; they are major pathogenic factors that cause different diseases and clinical outcomes. Clinically most important is *C. difficile*, which co-produces toxins A and B, both causally involved in antibiotic-associated diarrhoea and the severe form, pseudomembranous colitis (BARTLETT 1994; KELLY et al. 1994; KELLY and LAMONT 1998). Lethal toxin from *C. sordellii* is involved in diarrhoea and enterotoxaemia in domestic animals and in gas gangrene in man (HATHEWAY 1990). *C. novyi* α toxin has been identified as causative agent for gas-gangrene infections in man and animals (HATHEWAY 1990). The divergence between comparable cytotoxic features of the cytotoxin family and differences in clinical features may be due to the presence of additional pathogenic factors and the targeting of different organs by the toxin-producing bacteria. Here we will focus on the cytotoxic (i.e. in vitro) effects of the toxins.

Because toxin A induces fluid accumulation in the ileum of animals and ileal explants, it was designated as enterotoxin; this is in contrast to toxin B, which does not possess toxic activity towards animal ileum (LYERLY et al. 1985; TRIADAFILOPOULOS et al. 1987). Toxin B is about 100–1000-fold more cytotoxic to cultured cell lines and, therefore, has been identified as a cytotoxin (LYERLY et al. 1982). The cytotoxic activities of toxins A and B differ with respect to potency but not with respect to cytotoxic features. Both toxins induce shrinking and rounding of cultured cells, initially accompanied by formation of neurite-like retraction fibres. In the course of intoxication, the retraction fibres disappear, and the cells are completely rounded. In the terminal phase, the cells partially detach. The morphological changes are accompanied by disaggregation of the actin cytoskeleton. The cell-spanning stress fibres disappear, and the remainder of the actin filaments accumulate in the perinuclear space

Table 1. Family of large clostridial cytotoxins

Toxin	Synonym	Source
Toxin A	Enterotoxin, TcdA, ToxA	*Clostridium difficile*
Toxin B	Cytotoxin, TcdB, ToxB	*C. difficile*
Lethal toxin	TcsL, LT	*C. sordellii*
Haemorrhagic toxin	TcsH, HT	*C. sordellii*
α-Toxin	Tcnα	*C. novyi*

Table 2. Protein substrates and co-substrates of the large clostridial cytotoxins

Toxin	Molecular mass (kDa)	Co-substrate	Substrates and site of modification	Transferred moiety
Clostridium difficile toxin A	308	UDP-glucose	Rho(T37), Rac(T35), Cdc42(T35), Rap(T35)	Glucose
C. difficile toxin B	270	UDP-glucose	Rho(T37), Rac(T35), Cdc42(T35)	Glucose
C. sordellii lethal toxin	271	UDP-glucose	Rac(T37), Cdc42(T35), Ras(T35), Rap(T35), RalA(T46)[a]	Glucose
C. sordellii haemorrhagic toxin	~300	UDP-glucose	Rho(T37), Rac(T35), Cdc42(T35)	Glucose
C. novyi α-toxin	250	UDP-*N*-acetyl-glucosamine	Rho(T37), Rac(T35), Cdc42(T35), RhoG(T35)	*N*-acetyl-glucosamine

T, threonine; *UDP*, uridine diphosphate.
[a] The protein substrate specificity depends on the strain of *C. sordellii*.

(Ciesielski-Treska et al. 1989; Fiorentini et al. 1989, 1990, 1993; Malorni et al. 1991; Siffert et al. 1993). The other members of the family of large clostridial cytotoxins, i.e. α, lethal and haemorrhagic toxins, induce the same effects, with some variation in certain details (Popoff, 1987; Bette et al. 1991; Ciesielski-Treska et al. 1991; Oksche et al. 1992).

B. Structure of the Toxins

The cytotoxins are single-chained proteins with molecular masses from 250 kDa to 308 kDa (Table 2; Fig. 1). The proteins consist of three functional domains. The C-terminally located receptor binding domain covers about one third of the molecule. The intermediate part is hypothesised to possess a transmembrane domain, which is likely to mediate the translocation of the toxin into the cytosol. The enzymatic active domain, which catalyses the mono-

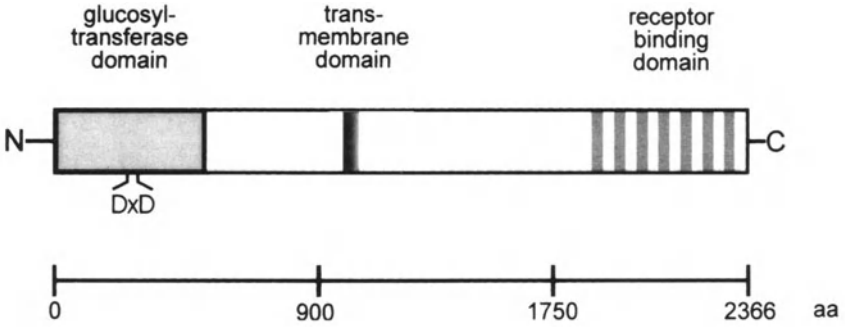

Fig. 1. Structure of *Clostridium difficile* toxin B. The structure of toxin B is depicted as an example for the homologous large clostridial cytotoxins. The toxins are constructed of three functional domains. The receptor-binding domain is located at the C-terminal part composed of repetitive peptide elements. The transmembrane domain located in the intermediate part is supposed to mediate the translocation of the toxin or a toxin fragment from acidic endosomal compartments into the cytosol. The N-terminal part covers the glucosyltransferase activity. The DXD motif is part of the catalytic domain responsible for the binding of Mn^{2+} and/or the co-substrate

glucosylation reaction, is in the N-terminal part of the protein (Von Eichel-Streiber 1993; Sect. D.II.2). Figure 2 gives the alignments of large clostridial cytotoxins.

Toxins A and B contain four conserved cysteine residues. Because alkylation by *N*-ethylmaleinimide does not alter toxicity, the cysteines are either not located at the surface of the molecule or are of no functional importance (Lyerly et al. 1986b; Shoshan et al. 1993). Amino acids 651–683 are reported to represent a putative nucleotide-binding site (Barroso et al. 1994; Lyerly and Wilkins 1995), but this region actually does not exhibit a classical consensus sequence for nucleotide binding.

The C-terminal part, the putative receptor-binding domain, is constructed of repetitive peptide elements (Chap. 15). Three findings have led to the notion that the C-terminal part is the receptor-binding domain: (1) the modular organisation of this region (Wren 1991; Von Eichel-Streiber et al. 1992b); (2) the homology to the carbohydrate-binding domain of streptococcal glycosyltransferases (Von Eichel-Streiber et al. 1992a, 1992b); and (3) the inhibition of the cytotoxic activity of toxin A by a monoclonal antibody recognising an epitope of the C-terminal domain (Lyerly et al. 1986a; Frey and Wilkins 1992). However, one finding is not reconcilable with this notion: the deletion of the C-terminal repetitive domain of toxin B decreases the cytotoxic potency only by a factor of ten (Barroso et al. 1994).

C. Cell Entry

Toxins A and B are thought to enter the cell via receptor-mediated endocytosis. Different cell lines show saturable binding for toxins A and B (Chaves-

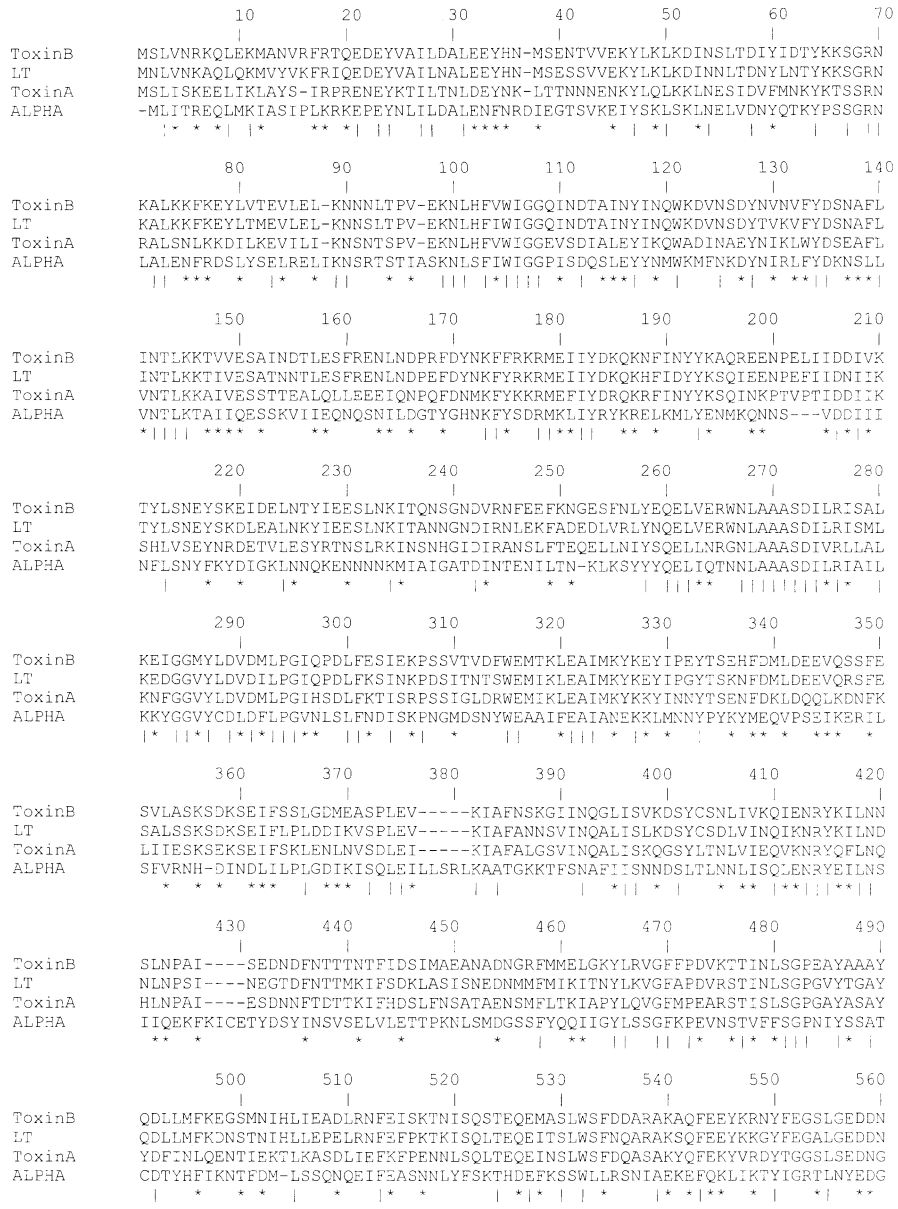

Fig. 2. Alignment of large clostridial cytotoxins (deduced from amino acid sequences). Toxin B from *Clostridium difficile*; *LT* (lethal toxin) from *C. sordellii*, toxin A from *C. difficile*; alpha (α toxin) from *C. novyi*. Sequences were aligned using CLUSTAL W (Thompson et al. 1994). Multiple alignment parameters: weight matrix = blosum; gap-opening penalty = 10.0; gap-extension penalty = 0.05. |, identity; *j*, high similarity

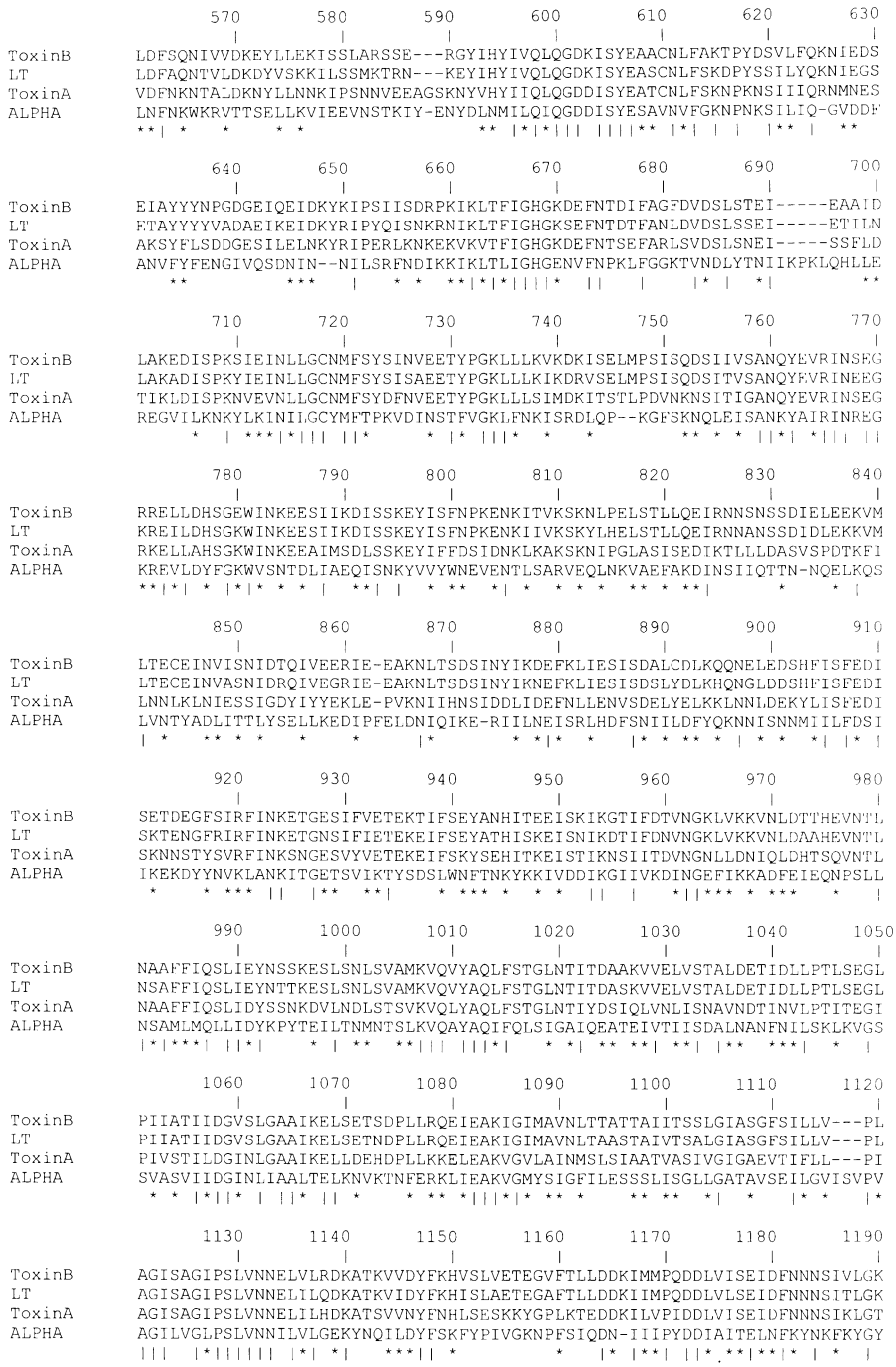

Fig. 2. *Continued*

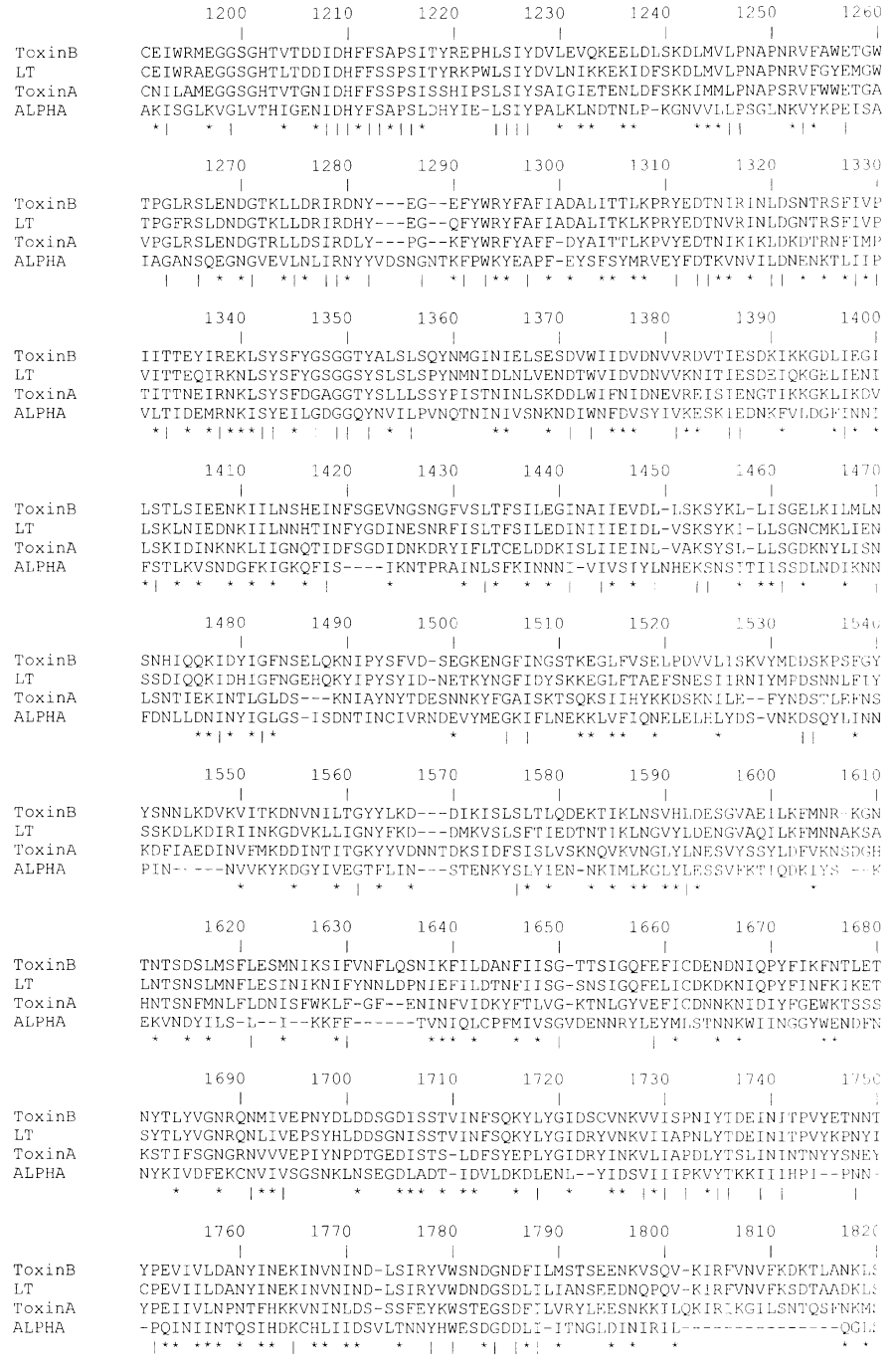

Fig. 2. *Continued*

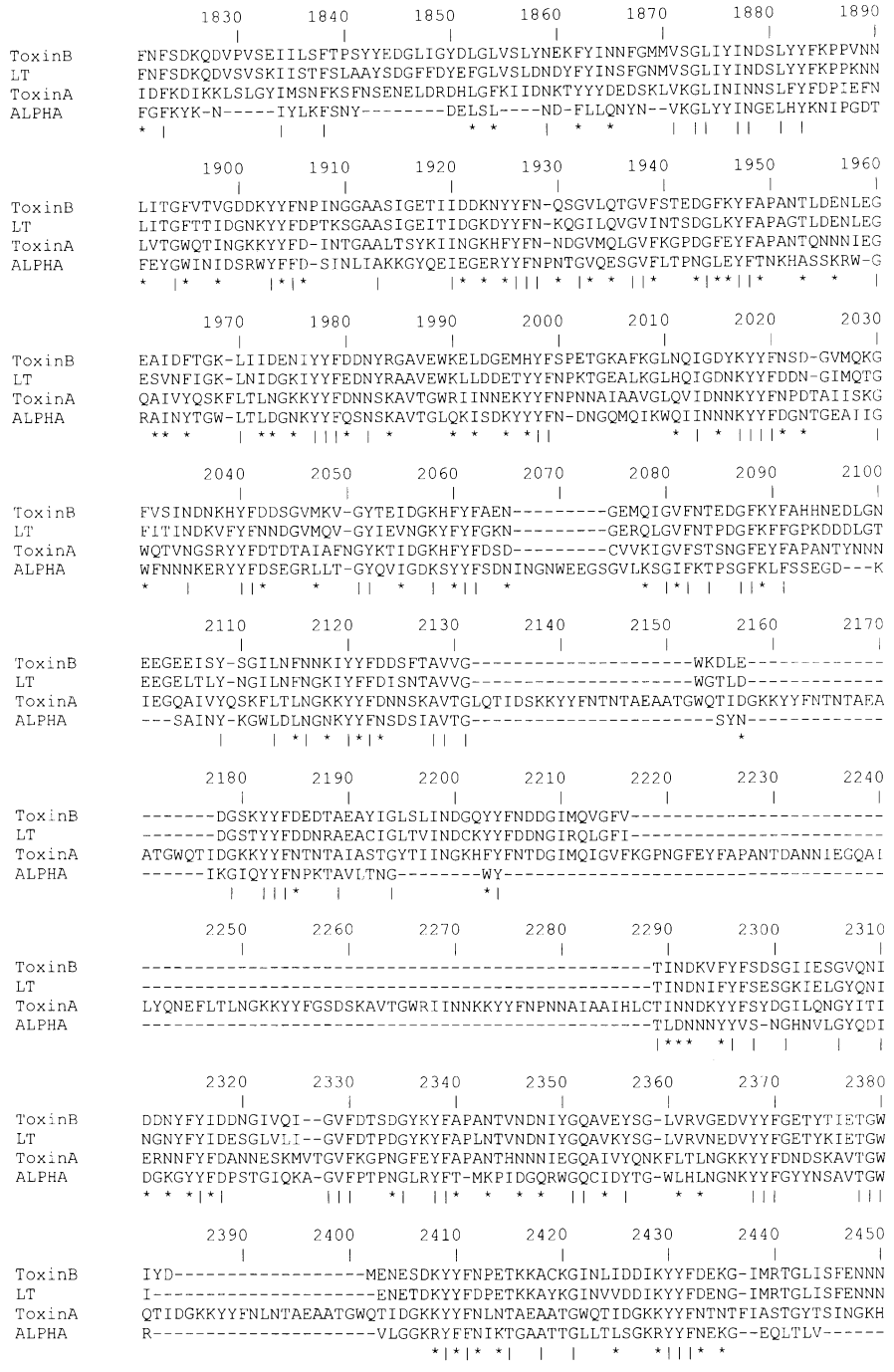

Fig. 2. *Continued*

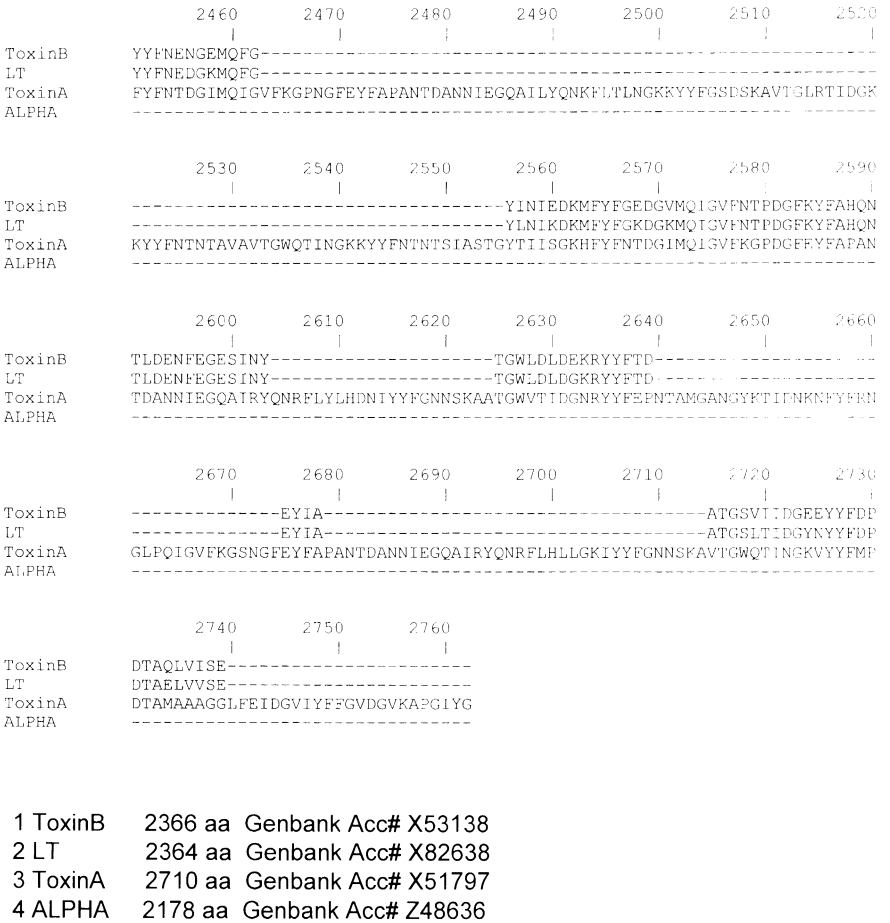

Fig. 2. *Continued*

Olarte et al. 1997). The binding sites seem to be different but have been determined with carbohydrates (Tucker and Wilkins 1991). Proteolytic treatment results in inactive toxins, indicating that the intact protein is essential for cell entry. Alkalinisation of acidic endosomal compartments inhibits the cytotoxic effects, which can be circumvented by short-term acidification of the extracellular medium. These findings are consistent with cell entry through receptor-mediated endocytosis and toxin release from acidic endosomes (Florin and Thelestam 1983, 1991; Henriques et al. 1987). The translocation process is proposed to be mediated through the insertion of the transmembrane domain of the toxins in the presence of low pH, a process analogous to the

cell entry of the well-characterised diphtheria toxin (KAUL et al. 1996). The intact cytotoxin molecule is necessary for cell entry, but it is unknown whether the complete protein or a proteolytically cleaved N-terminal fragment is translocated into the cytosol. If there is no proteolytical cleavage, the catalytic N-terminal part is likely to stay at the endosome after translocation through the transmembrane domain of the toxin.

It has been shown that toxin A binds to the terminal carbohydrate structure Galα1–3Galβ1–4GlcNAc, a finding from which it was concluded that the receptor is possibly a glycoprotein (KRIVAN et al. 1986; TUCKER and WILKINS 1991). This structure, however, is absent in humans (LARSEN et al. 1990). Recently, it was demonstrated that toxin A also binds to GalNAcβ1–3Galβ1–4GlcNAc, which is present in humans (KARLSSON 1995). More recently, the membranous sucrase–isomaltase glycoprotein was identified as a toxin-A receptor in rabbit ileal brush border (POTHOULAKIS et al. 1996). However, this receptor seems not to be of general importance, because it is not expressed in many toxin-sensitive tissues (human colon; POTHOULAKIS et al. 1996). The receptor of toxin B has not been characterised yet.

The repetitive structure of the receptor domains of toxin A and of toxin B argues for a modular organisation of this domain. With this modular structure, the toxin can bind to several possibly identical receptors to induce clustering followed by endocytosis. The current concept of toxin-A cell entry is that the toxin binds to its cellular receptor via a lectin-like binding.

Toxin A is believed to be the enterotoxin that disrupts the colonic epithelium to allow toxin B to act on non-epithelial tissue compartments. This notion is based on the findings that only toxin A is cytotoxic in animal models and in animal intestinal explants. Colonic epithelial cells from animals do not seem to possess a receptor for toxin B. However, recent findings suggest that human colonic epithelial cells are equally or even more sensitive to toxin B than to toxin A; this has significance for toxin B in human disease (HECHT et al. 1992; RIEGLER et al. 1995; CHAVES-OLARTE et al. 1997).

D. Molecular Mode of Action

I. Elucidation of the Molecular Mechanism of Action

One of the earliest observations was that the large clostridial cytotoxins induce depolymerisation of the actin filaments, resulting in morphological changes. The morphological and cytoskeletal alterations have been studied in detail, but the phenomenological studies did not lead to clarification of the mode of action. The observation that the treatment of cells with toxin A or toxin B resulted in a decrease in the adenosine diphosphate (ADP) ribosylation of the low-mass guanosine triphosphate (GTP)-binding protein RhoA was the first step in pinpointing the cellular targets and, finally, led to the identification of the toxins' mode of action.

C. botulinum exoenzyme C3 is a 25-kDa, single-chain mono-ADP-ribosyltransferase which selectively modifies RhoA, B and C proteins. The ADP–ribose moiety is linked to Asn-41, resulting in deactivation of the GTP-binding proteins (Aktories and Koch 1997). Intoxication of intact cells with toxin A or B resulted in a time- and concentration-dependent inhibition of the C3-catalysed ADP ribosylation of cellular Rho. Toxin A/B treatment, however, did not decrease the cellular amount of Rho (as detected in immunoblots excluding proteolytic degradation of Rho as the basis of inhibited ADP ribosylation; Just et al. 1994, 1995a). These findings rather indicated a stable modification of Rho; this modification prevented ADP ribosylation. The effect of toxin A/B on Rho was not restricted to intact cells but was also observed in cell lysates and in the respective cytosolic fractions. Recombinant Rho added to the cytosolic fraction was also affected by toxin A/B. The cytosol provided an essential cofactor that was of low molecular weight and was heat stable (Just et al. 1995a). This preliminary characterisation of the reaction was beneficial for producing sufficient amounts of modified recombinant Rho for analysis by mass spectrometry. The analysis revealed an increase in mass of 162 Da, consistent with the incorporation of a hexose. Biochemical studies identified the cofactor as uridine diphosphate (UDP)–glucose and the modification as glucosylation, i.e. incorporation of one glucose moiety (Just et al. 1995b, 1995c).

Toxins A and B are mono-glucosyltransferases which recruit UDP-glucose as a co-substrate to transfer the glucose moiety to the substrate protein (Fig. 3). The acceptor amino acid is threonine (Thr-37 in RhoA), consistent with an *O*-glucosylation type of reaction.

II. Enzymatic Activity

1. Co-Substrates

Toxin B is very potent and acts in picomolar concentrations. This early observation led to the notion that toxin B and toxin A possess enzymatic activity. The putative activity was elucidated by the identification of the type of modification induced by the toxins. Toxin A (Just et al. 1995b), toxin B (Just et al. 1995c), lethal toxin (Just et al. 1996; Popoff et al. 1996) and haemorrhagic toxin (Genth et al. 1996) use UDP–glucose, whereas α toxin (Selzer et al. 1996) recruits UDP–*N*-acetyl-glucosamine (UDP-GlcNAc) as its co-substrate (Table 2; Just et al. 1995b, 1995c, 1996). The activated nucleotide sugar is cleaved into glucose/GlcNAc and UDP, and the sugar moiety is transferred to the acceptor amino acid of the Rho proteins. Glucose/GlcNAc is bound to the hydroxyl group of the acceptor residue, threonine (Just et al. 1995b, 1995c; Popoff et al. 1996). One molecule of glucose is incorporated per molecule of Rho protein, consistent with a mono-glucosylation.

UDP–glucose and UDP–*N*-acetyl-glucosamine are recruited as co-substrates; other nucleotide sugars are not (Just et al. 1995b, 1995c). Many

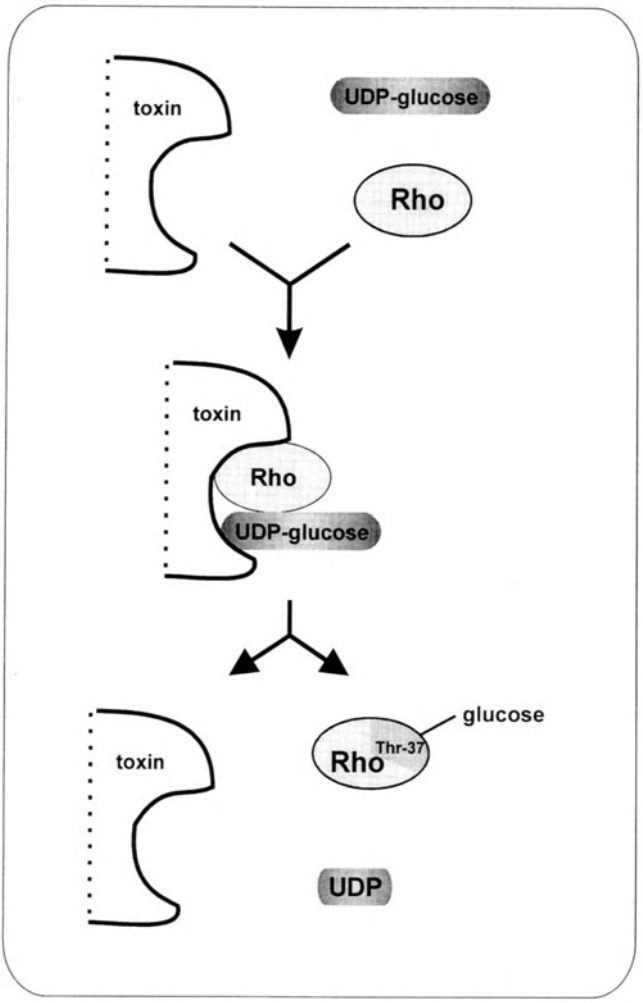

Fig. 3. Scheme of the glucosylation reaction. RhoA in the guanosine diphosphate-bound state forms a ternary complex with toxin B and the co-substrate uridine diphosphate (UDP)–glucose. The nucleotide sugar is cleaved, and the glucose moiety is transferred to threonine-37 of RhoA. The complex dissociates and releases glucosylated RhoA and UDP. Toxin B is ready to start a new glucosylation reaction. The glucosylation is, in principle, a reversible reaction; in the absence of UDP–glucose (but in an excess of UDP), toxin B deglucosylates RhoA to form UDP–glucose

types of nucleotide sugars are generated for further metabolism, but UDP–glucose is produced in the highest concentration, and the cellular concentration is estimated as 100 μM (Laughlin et al. 1988). Thus, the co-substrate is not a limiting factor for the intracellular enzyme activity of the toxins. That UDP–glucose is also the exclusive co-substrate also in intact cells has been demonstrated in a mutant Don cell line deficient in UDP–glucose. These cells are resistant to toxins A and B, but the resistance can be overcome by

supplementation with UDP–glucose through microinjection (Chaves-Olarte et al. 1996).

Although the catalytic mechanism of glucose transfer seems to be the same in toxins A and B, toxin B demonstrates an approximately 100-fold higher enzyme activity than toxin A (Chaves-Olarte et al. 1997). This magnitude of difference in activity between both toxins was also found in intact cells. When the toxins were microinjected to circumvent the cell-entry mechanism, the cytotoxic potency also differs by a factor of 100. Thus, the overall observed difference in the cytotoxic potencies of toxins A and B on intact cells can be explained by the mere difference in enzyme activity.

2. Catalytic Domain and Requirements for Catalysis

The three-domain structure of the large clostridial toxins has been deduced from sequence analysis (Chap. 15; Fig. 1). The biological activity has been assigned to the N-terminal fragment. Direct proof for this assignment has been obtained from the separate expression of the toxin fragments and their testing for glucosyltransferase activity. Only the N-terminal fragment of toxin B covering amino acids 1–900 showed glucosyltansferase activity, whereas the intermediary part (amino acids 901–1750) and the C-terminal fragment (1751–2366) were absolutely devoid of transferase activity (Hofmann et al. 1997). Successive C-terminal truncation of the N-terminal fragment led to a minimum fragment covering amino acids 1–546 of toxin B which exhibits full catalytic activity and which is fully cytotoxic when microinjected into intact cells (Hofmann et al. 1997). Further deletion resulted in an enzymatically inactive fragment (amino acids 1–516; Hofmann et al. 1997).

The active fragment shows binding to the co-substrate, detected by labelling it with azido-UDP–glucose; the inactive fragment does not bind UDP–glucose (Busch et al. 1998). The putative nucleotide-binding site (amino acids 651–683) is beyond the minimum catalytic fragment (amino acids 1–546) and is obviously not involved in the binding of UDP–glucose and the enzymatic activity.

In addition to the glucosyltransferase activity, the toxins exhibit glycohydrolase activity to hydrolytically cleave UDP–glucose (toxins A and B, lethal toxin) and UDP–GlcNAc (α toxin) into UDP and glucose/GlcNAc in the absence of protein substrates (Just et al. 1995c; Chaves-Olarte et al. 1997; Ciesla and Bobak 1998). The nucleotide specificity is identical with the transferase reaction; only UDP–glucose is hydrolysed (Just et al. 1995c). The glycohydrolase activity is about one to ten orders of magnitude lower than the transferase activity. The biological relevance of the glycohydrolase activity is unclear.

Surprisingly, the difference in hydrolase activity between toxin B and toxin A has been reported to be a factor of only five to six (Chaves-Olarte et al. 1997; Ciesla and Bobak 1998), whereas the difference in the transferase activity is of factor of 100 (Chaves-Olarte et al. 1997). Thus, the difference in the

transferase activity between toxin A and toxin B is likely to be based on the transfer of the glucose moiety to the acceptor protein rather than on the binding and cleavage of the co-substrate UDP–glucose. Toxins A and B should, therefore, differ in the K_m values of their protein substrates.

Because the glycohydrolase activity is a pure two-component system – consisting exclusively of UDP–glucose and toxin – and is thus devoid of other interfering factors, it is suitable to study the conditions of the catalytic activity. Glycohydrolase activity is stimulated by K^+ but inhibited by Na^+ (Ciesla and Bobak 1998). Thus, the hydrolase activity relies on specific effects of K^+ and not mere ionic strength (Ciesla and Bobak 1998). In view of the intracellular activity of toxins A and B, K^+ regulation of the enzyme activity makes sense. In addition to the monovalent cation K^+, glycohydrolase activity is strongly stimulated by Mn^{2+} and less by Mg^{2+}. The Mn^{2+} dependence has also been reported for the glucosyltransferase activity of lethal toxin, which shows about 90% homology to toxin B (Just et al. 1996). The divalent cation Mn^{2+} is an essential cofactor for several UDP–glucose hydrolases and many glycosyltransferases (McEuen 1992). These transferases possess a conserved DXD (aspartic acid–any amino acid –aspartic acid) motif surrounded by a hydrophobic region (Fig. 4; Wiggins and Munro 1998). DXD is very likely to

DXD motif

```
Toxin A   249 ELLNIYSQELLNRGNLAAASDIVRLLALKNFGGVYLDVDMLPGIHSDLFKTISRPSSI 306
Toxin B   250 ESFNLYEQELVERWNLAAASDILRISALKEIGGMYLDVDMLPGIQPDLFESIEKPSSV 307
α-Toxin   248 KLKSYYYQELIQTNNLAAASDILRIAILKKYGGVYCDLDFLPGVNLSLFNDISKPNGM 305
LT        250 DLVRLYNQELVERWNLAAASDILRISMLKEDGGVYLDVDILPGIQPDLFKSINKPDSI 307
Och1p     151 APVPIVIQAFKLMPGNILKADFLRYLLLFARGGIYSDMDTMLLKPIDSWPSQNKSWLN 208
Sur1p     102 EEYPWFLDTFENYKYPIERADAIRYFILSHYGGVYIDLDDGCERKLDPLLAFPAFLRK 159
Mnn1p     394 LDVSNTIHPKWRGDFGSYKSKWLVVLLNLLQEFVFLDIDAISYEKIDNYFKTTEYQKT 451
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Fig. 4. Alignment of the large clostridial cytotoxins and glycosyltransferases covering the DXD motif. *Clostridium difficile* toxins A and B, *C. novyi* α toxin, *C. sordellii* lethal toxin (*LT*), *Saccharomyces cerevisiae* Och1p mannosyltransferase, *S. cerevisiae* Sur1p mannosyltransferase, *S. cerevisiae* Mnn1p mannosyltransferase

be involved in Mn^{2+} binding (Wiggins and Munro 1998). This motif is also located in the N-terminal catalytic part of the large clostridial cytotoxins. Mutations of these conserved aspartic acids in lethal toxin (Asp286 and Asp288) completely abolish both glucosyltransferase and glycohydrolase activity (Busch et al. 1998). Furthermore, labelling of the N-terminal fragment of lethal toxin with azido-UDP–glucose is blocked in the DXD-deficient fragment even in the presence of high concentrations of Mn^{2+} (Busch et al. 1998). Thus, this motif represents a structure essential for catalysis. The aspartic acids are thought to participate in the coordination of Mn^{2+}, and Mn^{2+} allows correct positioning of the co-substrate UDP–glucose, which is then catalytically cleaved and transferred to the acceptor amino acid, threonine. The catalytic amino acid has not been identified yet.

3. Recognition of the Protein Substrates

For glucosylation, the toxin has to form a ternary complex with UDP–glucose and the substrate protein (Rho protein). Therefore, the toxins need – in addition to the UDP–glucose-binding site and the catalytic centre – a substrate-recognition site. Whereas toxin B exclusively modifies the Rho subfamily proteins, lethal toxin glucosylates Rac and Cdc42 from the Rho subfamily and Ras, Ral and Rap from the Ras subfamily (Sect. D.III.1). It is unlikely that there is only one single substrate-recognition site that is able to recognise variant combinations of the Rho and Ras GTP-binding proteins. Instead, it is conceivable that the toxins possess different recognition sites. This notion allows us to explain the differences in the substrate specificities of the isoforms of lethal toxin and the variant toxin of *C. difficile* (Table 3).

Because toxin B and lethal toxin are highly homologous (90%) but differ in their protein-substrate specificity (Sect. D.III.1.), chimeric toxins are helpful in restricting the site of substrate recognition. Testing the substrate specificity of various chimeras for the N-terminal parts of toxin B and lethal toxin led to the restriction and separation of recognition sites for the Rho and Ras proteins. Amino acids 408–468 in toxin B determine the specificity for Rho, Rac and Cdc42, whereas residues 364–408 in lethal toxin determine the specificity

Table 3. Protein substrate specificity of lethal toxin from various *Clostridium sordellii* strains

C. sordellii strain	Rho	Rac	Cdc42	Ras	Rap	Ral	Reference
CN6	(+)	+	(+)	+	+	+	(Just, unpublished data)
VPI9048	–	+	(+)	+	+	–	(Hofmann et al. 1996)
6018	–	+	–	+	+	+	(Just et al. 1996)
IP82	–	+	–	+	+	–	(Popoff et al. 1996)
C.d. B-1470	–	+	–	–	(+)	+	(Schmidt et al. 1998)

+, substrate; *(+)*, minor substrate; –, no substrate; *C.d.*, *C. difficile* (resembles *C. sordellii*).

for Rac and Cdc42 but not for Rho. Interestingly, lethal toxin and toxin B recruit different domains specific for the Rho proteins. The recognition of the Ras proteins is mediated from the region amino acids 408–516, which is adjacent to the Rho-recognition site (Hofmann et al. 1998). It seems that the substrate-recognition sites are modularly organised.

III. Cellular Targets of the Cytotoxins

1. Rho and Ras Proteins as Substrates

The substrate specificity was determined by testing recombinant low-molecular-mass GTPases for glucosylation by applying radioactively labelled UDP–glucose or UDP–*N*-acetyl-glucosamine. Toxin A and toxin B glucosylate RhoA, Rac1 and Cdc42, whereas other prototypes of the superfamily of low-molecular-mass GTPases, such as H-Ras, Rab5 and Arf1, are not substrates (Just et al. 1995b, 1995c). Both toxins seem to possess the identical protein-substrate specificity. Recently, however, it was reported that toxin A, but not toxin B, modifies Rap2 to a minor extent, indicating a difference in their targets (Chaves-Olarte et al. 1997; Table 2). α Toxin, which catalyses the incorporation of *N*-acetyl-glucosamine instead of glucose, has the same targets as toxin B, namely Rho, Rac and Cdc42 (Selzer et al. 1996).

The substrate specificity of lethal toxin, however, is not restricted to one GTPase subfamily. Furthermore, the specificity depends on the strain lethal toxin is purified from. Table 3 gives a synopsis of the substrates of the different known isoforms of lethal toxin (Hofmann et al. 1996; Just et al. 1996; Popoff et al. 1996). Toxin B from *C. difficile* strain 1470 resembles the lethal toxin and is, therefore, subsumed here (Schmidt et al. 1998).

The Rho and the Ras proteins are the preferred substrates for the large clostridial cytotoxins. The substrates were exclusively found by testing recombinant GTPases. In cell lysates, the cytotoxins catalyse incorporation of [^{14}C]glucose and [^{14}C]*N*-acetyl-glucosamine which are easy to detect by phosphorimaging. The cellular concentration of Rho is estimated at about 1 μM (Price et al. 1995), whereas other low-molecular-weight GTPases, such as Ras, are less concentrated in cells, and detection of [^{14}C]glucosylation is less sensitive. The diverse substrate specificity of the isoforms of lethal toxin clearly shows that testing a single prototype of the GTPase subfamily does not exclude the possibility that other members of the subfamily could be substrates. Thus, it is conceivable that the toxins recognise more substrates than identified so far.

2. Site of Modification

The glucose is transferred by the cytotoxins to threonine-37 in Rho. In Rac, Cdc42 and Ras, threonine-35 is equivalent to threonine-37 in Rho. The acceptor amino acids were determined by sequencing (Just et al. 1995b, 1995c), and the identified threonine-37 in Rho was corroborated by mutation (Just et al.

1995c). All large clostridial cytotoxins catalyse the modification of the identical acceptor threonine.

Threonine-37/35 is located in the effector domain of the low-molecular-mass GTPases, where coupling with the effector proteins takes place, resulting in downstream signalling. In addition, threonine-37/35 is involved in the coordination of Mg^{2+}, which binds to the β and γ phosphates of GTP. Exclusively in the GTP-bound state, this threonine participates in GTP binding. After hydrolysis and release of the γ phosphate, loop L2 moves, and the threonine-37/35 exposes its hydroxyl group to the surface of the protein (Pai et al. 1989, 1990; Wittinghofer et al. 1993). Thus, the hydroxyl group is accessible for glucosylation only in the GDP-bound form. Experimental data support this consideration. GDP-bound Ras is monoglucosylated, i.e. one mole of glucose is incorporated into one mole of Ras; Ras bound to GTPγS, the non-hydrolysable GTP analogue, is not a substrate (Herrmann et al. 1998). The recognition of the Rho and Ras proteins by the cytotoxins strictly depends on a defined conformational state and is not merely determined by the primary or secondary structures of the substrate.

3. Cellular Functions of the Rho Proteins

The Rho proteins belong to the Ras superfamily of low-molecular-mass GTP-binding proteins (GTPases), which serve as molecular switches in the intracellular signal transduction. These GTPases are characterised by their molecular weight (18–28 kDa), their C-terminal polyisoprenylation and their ability to bind and hydrolyse guanine nucleotides. They are inactive in the GDP-bound state, and binding of GTP induces activation, resulting in downstream signalling. The transition between inactive and active states is controlled by several regulatory proteins. The guanine-nucleotide-exchange factors (GEF) promote the exchange of nucleotides and binding of GTP. The GTPase-activating proteins (GAP) strongly stimulate the low intrinsic GTPase activity to terminate the activated state. The guanine-nucleotide-dissociation inhibitor traps the inactive GDP-bound form in a high-affinity complex. Binding of GTP induces changes in the conformation, which allows binding to the so-called effector protein. Effectors are often serine/threonine kinases, which possess a Rho-binding domain. Binding of Rho results in activation of the kinase (ROKα/Rho kinase), which phosphorylates downstream targets. In addition to kinases, Rho effectors also comprise multidomain proteins without enzymatic activity (rhotekin, rhophilin), which may serve as nuclei for multiprotein complexes that connect different signalling pathways. The signalling cycle of the Rho proteins is depicted in Fig. 5.

The best-characterised members of the Rho subfamily are Rho, Rac and Cdc42. In general, they are involved in the regulation of the dynamic actin cytoskeleton. Each of them, however, regulates distinct structures: Rho governs the formation of stress fibres and focal adhesions, Rac is involved in membrane ruffling and Cdc42 is involved in the formation of filopodia. The

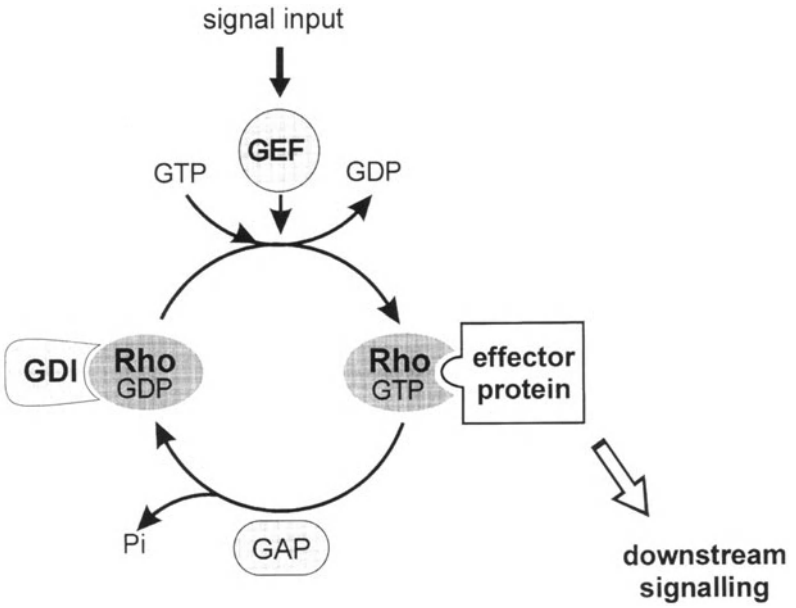

Fig. 5. Guanosine triphosphate (*GTP*)ase cycle of Rho. Inactive Rho is guanosine diphosphate (*GDP*)-bound and complexed with GDI (guanine-nucleotide dissociation inhibitor). Activation of Rho is mediated by GEFs (guanine-nucleotide exchange factors), which promote binding of GTP to Rho and translocation from the cytosolic to the membrane compartment. Here Rho–GTP interacts with effector proteins (serine/threonine kinases or adapter proteins), which transmit the signal downstream. The active state is terminated by GAPs (GTPase-activating proteins), which strongly increase the intrinsic GTPase activity to result in inactive GDP-bound Rho. GDP-bound Rho is extracted by GDIs from the membrane and complexed as an inactive Rho pool in the cytosol

best-understood functional module is the formation of the stress fibres: Rac and Rho regulate the phosphatidyl-inositide (4)-phosphate-5-kinase (PI-5-kinase) to form phosphatidyl-inositide-4,5-phosphate (PIP_2). PIP_2 stimulates actin polymerisation and filament growth through interaction with several actin-binding proteins (gelsolin, profilin). The stress fibres, a supra-organisation of actin filaments, are governed by the RhoA-dependent Rho-kinase, which phosphorylates the myosin light chain, thereby activating the actin–myosin system in non-muscle cells. The membrane attachment of the stress fibres is managed through the ERM (ezrin/radixin/moesin) proteins. These bifunctional proteins bind through their N-terminal part to transmembrane proteins (CD44 or intercellular-adhesion-molecule proteins) and interact (through their C-terminal part) with the actin filaments. This interaction is essential for Rho-governed cytoskeletal changes.

The Rho proteins are involved in several cellular events. Table 4 gives a short overview of their multiple functions.

Table 4. Cellular functions of the Rho proteins

Cellular process/location	Detailed functions of Rho
Organisation of the actin cytoskeleton	Stress fibres, membrane ruffling, filopodia formation, cell adhesion, cell–cell contact, cell morphology, cell motility
Membrane trafficking	Endocytosis, exocytosis, phagocytosis
Smooth-muscle contraction	Calcium sensitisation
Phospholipid metabolism	PI-5-kinase, PLD, PLC
Cell-cycle progression	Transition from G1 to S phase
Reactive oxygen species	NADPH oxidase of neutrophils
Transcriptional activation	JNK, *p38RK*, NFκB
Cell transformation, apoptosis	Co-operation with the proto-oncogene Ras

JNK, c-Jun *N*-terminal kinase; *NADPH*, reduced nicotinamide adenine dinucleotide phosphate; *NFkB*, nuclear factor κB; *PI-5-kinase*, phosphatidyl-inositide (4)-phosphate-5-kinase; *PLC*, phospholipase C; *PLD*, phospholipase D.

Rho/Rac/Cdc42-dependent signal pathways are stimulated by receptor-tyrosine kinases (platelet-derived growth factor for Rac) and by G-protein-coupled receptors (lysophosphatidic acid for Rho, bradykinin for Cdc42). The regulation of the exchange factors (GEFs) that directly promote activation of the Rho proteins is quite unclear, but there are some data indicating that tyrosine phosphorylation or phospholipid (PIP_2) binding is involved. The functional hierarchy of Rho proteins (Cdc42 activates Rac, and Rac activates Rho) and the crosstalk between the proto-oncogene Ras and Rac is also part of the upstream regulation of the Rho proteins (Machesky and Hall 1996; Narumiya 1996; Ridley 1996; Tapon and Hall 1997; Van Aelst and D'Souza-Schorey 1997; Amano et al. 1998; Hall 1998; Mackay and Hall 1998; Sasaki and Takai 1998).

VI. Functional Consequences of Glucosylation

1. Consequences on the GTPase Cycle

The cellular functions of the low-molecular-mass GTPases of the Rho and Ras subfamilies are determined by their ability to cycle between the inactive and active states, a process which is regulated by various regulatory proteins (Sect. D.III.3). Because the Ras GTPase cycle is characterised very well, all aspects of the functional consequences of glucosylation were studied with recombinant proteins (Herrmann et al. 1998). The activation of Ras by exchange factors (GEFs) is decreased but not completely inhibited. Coupling to the effector protein Raf-kinase is completely blocked. The activity of intrinsic GTPase is reduced, but the GAP-stimulated GTPase is completely inhibited. No regulatory protein of the Ras cycle is sequestered, arguing against a dominant negative mode of action.

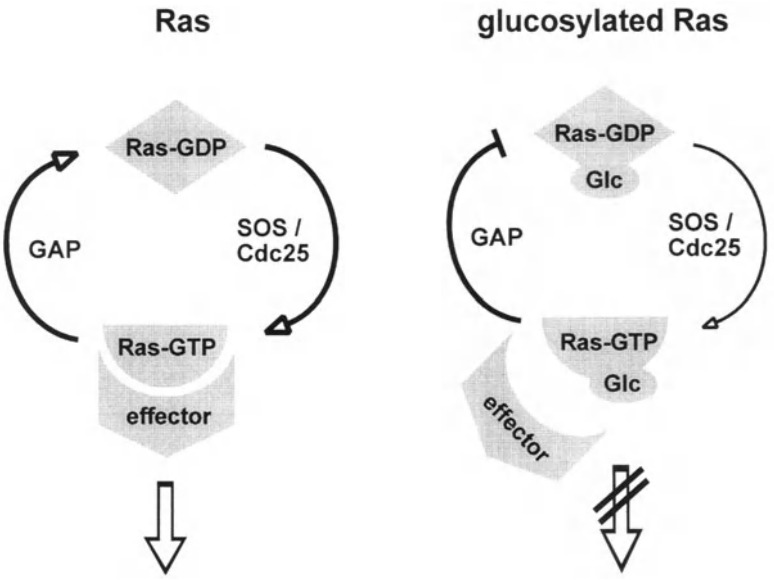

Fig. 6. Guanosine triphosphate (GTP)ase cycle of unmodified and glucosylated Ras. The left panel gives the GTPase cycle of Ras. Inactive guanosine diphosphate (GDP)-bound Ras is activated by exchange factors, such as Sos or Cdc25. Ras–GTP binds to the effector proteins, e.g. Raf kinase, which are activated to transmit the signal downstream. The active state is terminated by GAPs (GTPase-activating proteins), which promote inactivation of Ras. The *right panel* shows the cycle of glucosylated Ras. Ras, in the GDP-bound form, is mono-glucosylated at threonine-35. Loaded with GTP catalysed by Sos or Cdc25, Ras is slowed down but not inhibited. Because it is unable to interact with GAP, glucosylated Ras is enriched in the GTP-bound form. However, interaction with effector proteins, such as Raf kinase is fully blocked, resulting in a complete inhibition of downstream signalling

Comparable data were found for glucosylated RhoA, except that interactions with exchange factors were not studied (Sehr et al. 1998). Thus, the crucial step of downstream signalling, – effector coupling – is completely inhibited. Figure 6 shows a schematic model of the influence of glucosylation on the GTPase cycle of Ras.

2. Biological Consequences

The actin-depolymerising activity of the cytotoxins can be explained by the inactivation of Rho. The serine/threonine kinase ROK, which is stimulated by activated RhoA, directly phosphorylates the myosin light chain (MLC) to enhance formation of stress fibres. Inactivation of MLC by MLC phosphatase is blocked by ROK-catalysed phosphorylation of the myosin-binding protein. A decrease in ROK activity by inactivation of Rho results in an increased activity of phosphatases, which finally leads to dephosphorylation of MLC and subsequent depolymerisation of stress fibres. The growth of actin filaments is

regulated by actin-binding proteins whose activity is stimulated by PIP_2 formed by the Rho/Rac-regulated phospholipid kinase. Inactivation of Rho/Rac leads, through decrease in PIP_2 formation, to an impaired F-actin stability. The actin cytoskeleton is dynamically regulated by polymerising and depolymerising signals. Shut-down of the polymerising signals by the large clostridial cytotoxins leads to the predominance of depolymerising inputs.

The primary target tissue of the *C. difficile* toxins is the colonic epithelium. Human colon carcinoma (T84) cells are polarised in culture and form tight junctions and, therefore, they are an established model for the colonic epithelial barrier. Toxins A and B disrupt the barrier function by opening the tight junctions (Triadafilopoulos et al. 1987; Hecht et al. 1988; Moore et al. 1990; Hecht et al. 1992). This effect is not merely caused by the breakdown of actin filaments but by inactivation of the ability of Rho to regulate tight-junction complexes (Nusrat et al. 1995; Jou et al. 1998). This barrier-disrupting effect of toxins A and B is supposed to increase colonic permeability, the basis of the watery diarrhoea, which is a typical feature of *C. difficile*-induced antibiotic-associated diarrhoea.

Toxins A and B have been reported to induce apoptosis (Mahida et al. 1996; Calderon et al. 1998; Fiorentini et al. 1998), consistent with the function of Rho in the apoptotic process (Gomez et al. 1998). Apoptosis is induced by many signals, and one is the detachment of cells form their extracellular matrix (Ruoslahti 1997). The cytotoxins, which are known to induce detachment through their actin-filament-disrupting properties, induce apoptosis in a manner similar to the treatment of cells with ethylene diamine tetraacetic acid or neutrophil elastase (Shibata et al. 1996).

In addition to accessory effector domains, the domain covering amino acids 30–42 is thought to be an essential effector domain for downstream signalling, and a glucose moiety is likely to inhibit all signal pathways downstream of Rho and Ras. The cytotoxic effect is easy to observe and has led to its classification as a cytotoxin. However, the depolymerisation of the actin-filament system is only one of many cellular responses to the inactivation of Rho, Rac and Cdc42. For example, regulation of endocytosis in *Xenopus* oocytes (Schmalzing et al. 1995), exocytosis of mediators from rat basophilic leukaemia (RBL) cells (Prepens et al. 1996) and the regulation of phospholipase D (Schmidt et al. 1998) are impaired by toxin B independently of the cytoskeletal effects.

Because glucosylation does not inhibit one single Rho function but inhibits many or even all functions, overlapping or unexpected findings after cell intoxication are observed; destruction of the actin cytoskeleton of RBL cells by cytochalasin D or the actin ADP–ribosylating *C. botulinum* C2 toxin results in an increase of stimulated degranulation (exocytosis). However, intoxication with toxin B, which also leads to disruption of the actin filaments, inhibited stimulated degranulation – the opposite effect was expected (Prepens et al. 1996). Direct regulation of exocytosis by Rho is dominant over the influence of the actin filaments, which are also governed by Rho. The large

clostridial cytotoxins can no longer be classified as simple cytoskeleton-disrupting toxins; they interfere with many vital functions of the cell, especially those assigned to the Rho and Ras proteins.

E. Concluding Remarks

The large clostridial cytotoxins cause comparable morphological and cytoskeletal changes that are mediated by their intrinsic enzymatic ability to inactivate Rho proteins, the regulators of the actin cytoskeleton. Comparable to other enzymatically active exotoxins, the large cytotoxins recruit ubiquitiously available cofactors (nucleotide sugars) to alter pivotal regulators of the cell machinery. At the moment, it is unclear why the toxins need 200–250 kDa of protein to target their enzymatic domain (60 kDa) to the cellular substrates. Maybe an additional biological activity is concealed in this part of the molecule, or the toxins have evolved a sophisticated targeting machinery that makes almost all cells sensitive to the toxins.

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CHAPTER 15

Molecular Biology of Large Clostridial Toxins

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A. Introduction

The clostridia are well known for the production of extracellular toxins and exoenzymes, many of which are involved in human and animal disease. The largest of the clostridial toxins are those produced by *Clostridium difficile*, *C. sordellii*, and *C. novyi*. These toxins, which include toxin A [molecular weight (M_r) = 308 000 Da] and toxin B (M_r = 270 000 Da) of *C. difficile*, toxin HT (hemorrhagic toxic; M_r = 300 000 Da) and toxin LT (lethal toxin; M_r = 260 000 Da) of *C. sordellii*, and α-toxin (M_r = 250 000 Da) of *C. novyi*, are all lethal and cytotoxic. In addition to having similar biological properties, they also display immunological cross-reactivity. Thus, BETTE et al. recently suggested that they comprise a group of closely related toxins (BETTE et al. 1991). Research within the past few years on the genetics and enzymatic activity of these toxins has established that these toxins do indeed belong to a distinct group. They not only share extensive sequence identity, they also cause cytotoxicity by similar molecular mechanisms. Accordingly, they are now referred to collectively as the large clostridial cytotoxins. This chapter will discuss the current state of knowledge regarding the molecular biology of these toxins. Due to its importance as a human pathogen, the majority of research has been done with toxins A and B of *C. difficile*.

C. difficile was an almost unknown species of bacteria until it was implicated as the cause of pseudomembranous colitis (PMC). Hall and O'Toole first isolated the organism from healthy newborn infants in 1935 and named it *Bacillus difficilis* after the apparent difficulty they encountered in isolating it (HALL and O'TOOLE 1935). The organism was shown to produce a toxic culture filtrate, which was lethal to animals upon injection. SNYDER (1937) confirmed and extended these findings and further showed that the toxic activity was neutralized by antisera to *C. difficile* (SNYDER 1937). Few studies followed its initial discovery, perhaps due to the fact that the organism did not appear to cause disease.

Things changed dramatically in 1977, as *C. difficile* became implicated as the cause of a lethal colitis that resulted from treatment with antibiotics. PMC was first called clindamycin colitis, because this new (at the time) antibiotic was the first to be associated with this severe form of antibiotic-associated colitis. When clindamycin colitis was first observed, no one had any idea that

it was caused by a clostridium. Several groups of investigators started work simultaneously on the disease (Bartlett and Gorbach 1977; Bartlett et al. 1977, 1978a; Larson et al. 1977a; Larson 1977b, 1978; Bartlett 1978b; Chang et al. 1978a, 1978b; George et al. 1978a, 1978b; Allo et al. 1979; Rolfe and Finegold 1979; Taylor and Bartlett 1979; Willey and Bartlett 1979; Rehg 1980). They soon found that hamsters also died from colitis several days after clindamycin was administered. The fecal material from both the humans and hamsters was toxic to tissue-culture cells, and this suggested to some researchers that a cytopathic virus was causing the problem. They found that, unlike the case with viruses, the toxicity could not be propagated or grown but instead merely diluted out as the contents were diluted. This was more suggestive of a toxin. The real breakthrough occurred when they found that multivalent clostridial antisera for gas gangrene neutralized all of the effects of the fecal filtrates on tissue-cultured cells. The active ingredient in the mixture was antitoxin to *C. sordellii* toxins, so they tried to isolate this organism from the feces – with no success. *C. difficile* had been isolated previously from many patients, but it had been ignored because it was non-pathogenic. When culture filtrates from all the clostridial isolates were tested, the *C. difficile* filtrates were cytotoxic and were neutralized by the *C. sordellii* antitoxin. We now know that this cross-neutralization is the result of the production of very similar toxins by both species.

C. difficile causes PMC, which is the most serious of a spectrum of antibiotic-associated diarrheas. Only about 15–20% of antibiotic diarrheas are caused by *C. difficile*; the mildest forms are osmotic diarrheas, and others may be caused by other microbes, including *C. perfringens* (Borriello 1995). *C. difficile* is an opportunistic pathogen which can grow in the human colon only when the normal flora have been drastically reduced by antibiotic therapy. The organism is ingested as spores that hatch in the intestine and grow in the absence of competition from the flora. The toxins are produced as the growth rate of the organism slows, resulting in both destruction of much of the colonic epithelium and massive diarrhea. The diarrhea keeps the normal flora from becoming re-established, and the destruction of the colonic mucosa can result in death of the patient from shock. The hospital ward becomes heavily contaminated with the spores, and the next patients are at increased risk of infection. Over 300,000 cases are diagnosed in the U.S. each year. Even when correctly diagnosed and treated, the disease adds 1–2 weeks and US $10000 to the hospital stay. When the costs of diagnosis, therapy, and extended hospital stays are calculated, this disease adds about a billion US dollars to healthcare costs every year in the U.S. alone.

Research was initially focused on the potent cytotoxic activity, and the tissue-culture assay was used to follow the toxin through purification. The toxic activity eluted as a single peak in the large-molecular-weight fractions from size-separation columns, and almost all of the cytotoxicity eluted as a single peak from ion-exchange columns. Two groups, one at Johns Hopkins in the U.S. (Taylor and Bartlett 1979) and one at Gifu University in Japan (Banno

et al. 1981) noticed that, whereas the cytotoxic fractions from the size-separation column were positive in the rabbit-ileal-loop test for diarrheal toxins, the main cytotoxic peak from the ion-exchange column was not. A second, very small peak of cytotoxicity that eluted much earlier from the column had all of the ileal loop activity, so there were two toxins. John Bartlett's group called these A and B, whereas BANNO et al. called them D-1 and D-2. Toxins A and B became the accepted names. Both are cytotoxic, but toxin B is much more active in the majority of tissue-cultured cells. Toxin A appears to be less active, because the carbohydrate receptors for toxin A are only present in large numbers on colonic epithelial cell lines. The receptor for toxin B has never been identified, but it must be ubiquitous, since essentially all cell lines are very susceptible. Toxin A has been shown to be a powerful enterotoxin and causes the initial damage to the colonic epithelium, whereas toxin B is not active in enterotoxin assays but seems to exacerbate the damage caused by toxin A. Hamsters receive some protection from antibodies to toxin A, but the best protection is achieved with antibodies or vaccines to both toxins (FERNIE et al. 1982). Recently, the situation has become more complicated, with some unusual strains producing toxins that have some characteristics of both A and B. These unusual toxins are more similar to the related toxins of *C. sordellii*.

C. sordellii and *C. novyi* belong to a group of pathogens termed histotoxic clostridia, which cause the tissue destruction associated with gas gangrene (myonecrosis). *C. sordellii* was first described by Sordelli who, in 1922, isolated several strains from wound infections. *C. novyi* was first recognized in 1894 by Novyi. The principal habitat of the organisms appears to be soil, and they cause a variety of infections in domestic livestock, including wound infections, peritonitis, and systemic infections often associated with the presence of liver flukes, which are thought to carry spores to the liver, where they germinate. *C. sordellii* is rarely associated with human infection, although infections can occur when wounds are exposed to contaminated soil. *C. novyi*, on the other hand, is a fairly common cause of gas gangrene in humans (ARSECULERANTE et al. 1969). It has been the cause of death for many soldiers whose wounds became contaminated with soil. During World War II, for example, it was isolated in 42% of gas-gangrene cases in one study (SMITH 1984).

B. Purification and Characterization of Large Clostridial Toxins

I. Toxin Production

A variety of media have been used for the growth of *C. difficile* and production of the toxins (ROLFE and FINEGOLD 1979; TAYLOR and BARTLETT 1979; SULLIVAN et al. 1982; HASLAM et al. 1986; SEDDON and BORRIELLO 1989). Recently, KAZAMIAS et al. produced unusually high cytotoxicity titers by growth in alkaline trypticase yeast-extract media supplemented with manni-

tol (Kazamias and Sperry 1995). The toxins are produced and released post-exponentially near the beginning of the decline phase of the bacterial growth cycle (Ketly et al. 1984). There is no correlation of toxin production, however, with sporulation, as occurs with *C. perfringens* enterotoxin (Ketly et al. 1986).

Regardless of the growth medium used, only modest amounts of toxin can be produced in free-standing anaerobic cultures. Much higher levels of the toxins are produced when *C. difficile* is grown in dialysis-sac cultures. The method, which was originally developed by Sterne and Wentzel for the production of botulinum toxin, works quite well for the production of toxins A and B (Sterne and Wentzel 1950). In this method, the organisms are grown inside a dialysis sac that has been suspended in a rich media, such as brain–heart infusion broth. The organisms grow slowly and reach a very high density within the dialysis sac after about 3 days. The toxins are released within the sac and, therefore, are concentrated in a small volume. Moreover, the membrane of the dialysis sac excludes any high-molecular-weight material from the culture filtrate. This eliminates any interference by media components during purification. The dialysis-sac-culture method has been used routinely to produce culture filtrate for purification of toxins A and B.

Toxins A and B appear to be coordinately regulated, since toxigenic strains almost invariably produce either high or low levels of each toxin. The level of toxins produced varies by as much as 100 000-fold among toxigenic *C. difficile*. As a result, the isolation and maintenance of a highly productive toxigenic strain was an important step in research on the toxins. The most extensively studied toxigenic strain has been VPI 10463 (also ATCC 43255), from which toxins A and B were characterized by Sullivan et al. (1982). This strain, originally isolated from a wound, consistently produces high levels of both toxins and has been used for most studies on the biological and molecular properties of toxins A and B.

The toxins of *C. sordellii* and *C. novyi* are also produced in large amounts when grown in dialysis-sac cultures, whereas much smaller amounts of the toxins are made during rapid growth in free-standing cultures. This suggests that their expression may be controlled by environmental signals similar to those controlling *C. difficile* toxins. Culture filtrates from dialysis sacs have also been used for purification of LT and HT from *C. sordellii* and α-toxin from *C. novyi*.

II. Purification and Physicochemical Properties

1. *C. Difficile* Toxins

Numerous methods have been used to purify toxins A and B from *C. difficile* culture filtrate (Rolfe and Finegold 1979; Taylor and Bartlett 1979; Banno et al. 1981, 1984; Sullivan et al. 1982; Rihn et al. 1984; Lyerly et al. 1986; Pothoulakis et al. 1986; Krivan and Wilkins 1987). Despite their similarity, the toxins behave very differently in salting-out steps and on ion-exchange-

chromatography resins. In general, due to the similar sizes of the toxins, purification requires careful attention to the separation of toxins A and B. In the case of toxin B, it is particularly difficult to eliminate the enolase enzyme. Enolase of *C. difficile* is a 50000-Da protein that has an elution profile similar to that of toxin B on anion exchange. Moreover, under native conditions, it forms a large-molecular-weight multimeric protein. Thus, toxin-B preparations purified by anion exchange are often contaminated with enolase. The contaminating enzyme caused some confusion in previous work on the mechanism of toxin B, which was erroneously reported to be due to enolase activity (Fluit et al. 1990; Knoop et al. 1990). Toxin A is more resistant to extremes of pH and to proteases than toxin B (Lyerly et al. 1986). Both toxins can be protected by reducing agents and are inactivated by oxidizing agents.

2. *C. Sordellii* and *C. Novyi* Toxins

Both toxins from *C. sordellii* have been purified to homogeneity. Popoff purified LT from *C. sordellii* by ion exchange and gel filtration (Popoff 1986). The M_r was estimated at 250000 Da, with a pI of 4.5. LT was lethal for mice, cytotoxic, and induced edema upon intradermal injection. Antisera specific for LT neutralized its biological properties and those of *C. difficile* toxin B. Martinez purified HT from *C. sordellii* by immunoaffinity chromatography using a monoclonal antibody (PCG-4) to *C. difficile* toxin A, which cross-reacts with HT (Martinez and Wilkins 1988). The toxin had an estimated M_r of 300000 Da with an apparent pI of 6.1. It was cytotoxic, lethal, and elicited an accumulation of hemorrhagic fluid in rabbit ileal-loop assays. Antisera to *C. difficile* toxin A neutralized HT biological activities and reacted by immunoblot and immunodiffusion. The N-terminus of HT was nearly identical with that of toxin A. These studies established that the physicochemical and biological properties of *C. sordellii* LT and HT are similar to those of *C. difficile* toxins B and A, respectively (Martinez and Wilkins 1992).

C. novyi α-toxin has been purified by anion exchange and has an apparent M_r of 200000 Da and a pI of 5.8 (Bette et al. 1989, 1990, 1991). It is also cytotoxic, lethal, and produces edema. α-Toxin does not cross-react, nor is its cytotoxicity neutralized, with antibodies to the other large clostridial toxins. Thus, α-toxin appears to be more distantly related to *C. difficile* toxins A and B than LT and HT of *C. sordellii*.

III. Biological Properties

1. *C. Difficile* Toxins

Both toxins A and B cause similar rounding of tissue-culture cells, and toxin B is active against every cell line that has been tested. Toxin A is far less cytotoxic in most cell lines compared with toxin B. When tested on certain cell lines, however, particularly those of intestinal origin, such as HT-29, its activity approaches that of toxin B (Tucker et al. 1991). This is presumably due to

an increased density of carbohydrate receptors for toxin A in these cell lines. Rounding of intoxicated cells is accompanied by disassembly of the actin microfilaments, whereas microtubules and intermediate filaments are only secondarily affected (Thelestam and Bronnegard et al. 1980; Mitchell et al. 1987; Ottlinger and Lin 1988; Fiorentini et al. 1990). Although the microfilament cytoskeleton is preferentially affected, actin itself is not the target. Rearrangement of actin and the actin-binding proteins vinculin and talin has been reported. Recent breakthroughs have revealed the molecular mode of action by which these toxins cause disassembly of the actin cytoskeleton (see below and Chap. 14).

Toxin A is an extremely potent enterotoxin, with microgram amounts causing fluid secretion in animal loops (Libby et al. 1982; Lyerly et al. 1982; Lima et al. 1988; Lyerly and Wilkins 1995). In fact, on a molar basis, it is as active as cholera toxin in the rabbit ileal-loop assay for enterotoxic activity. Its mechanism of action, however, is quite different. Unlike cholera toxin, toxin A causes extensive damage to the epithelial lining of the intestine. The villus tips of the epithelium are initially disrupted, followed by damage to the brush-border membrane. The mucosa eventually becomes denuded, accompanied by extensive infiltration with inflammatory neutrophils. The massive inflammation undoubtedly plays an important role in pathogenesis. The fluid response is due, in part, to damaging of the integrity of the intestinal barrier. The cytotoxic activity of toxin A also results in disruption of the tight junctions, and this may play a role in the initial fluid loss (Hecht et al. 1988; Hippensteil et al. 1997). For example, the disruption of tight junctions is responsible for the enterotoxicity of the zonula occludens toxin (ZOT) of *Vibrio cholera* and fragilysin, the metalloprotease enterotoxin of *Bacteroides fragilis* (Fasano et al. 1991; Moncrief et al. 1995).

Despite the fact that toxin B is a much more potent cytotoxin than toxin A, it does not cause damage or a fluid response when injected alone in intestinal loops (Lyerly et al. 1982; Lima et al. 1988; Lyerly and Wilkins 1995). The lack of enterotoxic activity by toxin B may be due to an inability to bind to a receptor on the intestinal brush-border membrane cells under normal physiological conditions. Thus, toxin A, which is able to tightly bind to specific carbohydrate receptors on the surface of intestinal cells, initiates damage to the intestine. Toxin B then contributes to extensive damage during the course of disease once it gains access to the underlying tissue. This role for toxin B in pathogenesis is supported by experiments on the effects of the toxins in the hamster intestine (Lyerly et al. 1985). In these studies, toxin B, given intragastrically to hamsters, did not cause a response, whereas toxin A caused death by this route. However, when toxin B was given along with sub-lethal amounts of toxin A, the animals died with no observable intestinal pathology. Moreover, if the intestine is physically disrupted, toxin B gains access to the systemic circulation and causes death. There remains controversy about the role of the toxins in human disease. It has recently been observed that toxin B is more potent than toxin A to the human colonic epithelium in vitro (Riegler

et al. 1995). Whether or not this correlates with what happens in the intact human intestine remains to be determined.

Both toxins are lethal when injected intraperitoneally or subcutaneously in small amounts (EHRICH 1982; LYERLY et al. 1982; LYERLY and WILKINS 1995). Contrary to the cytotoxic activity of the toxins, the lethal activity of toxin A is as potent as that of toxin B, with both toxins having a minimum lethal dose of about 50 ng in mice. The mechanism by which the toxins cause death when administered systemically is unknown. In mice injected intraperitoneally, there is some evidence of liver damage. ARNON et al. examined the effects of the toxins in rhesus monkeys and noted that the animals apparently died from cessation of breathing (ARNON et al. 1984). Death is not accompanied by the paralysis characteristic of the botulinum and tetanus neurotoxins.

In addition to their direct effects on cells, the toxins have been shown to elicit the production of various biological-response modifiers, such as cytokines and neurokinins, which undoubtedly play a significant role in the pathogenesis of *C. difficile* infections. The release of these chemoattractants may account for the extensive infiltration of neutrophils at the site of *C. difficile* infections, with the resulting extensive inflammatory tissue damage (MAHIDA et al. 1996; LINEVSKY et al. 1997; MELO et al. 1997; ROCHA et al. 1997; CALDERON et al. 1998; CASTAGLIUOLO et al. 1998a). Induction of apoptosis by toxin A is associated with the release of cytokines by monocytes and intestinal epithelial cells (MAHIDA et al. 1996). In 1994, PATHOULAKIS et al. observed that the enterocolitis induced by toxin A in rat ileal loops was inhibited by a receptor antagonist of neurokinin-1, the receptor for substance P (PATHOULAKIS 1994). Moreover, toxin A induces secretion of substance P in animal intestinal loops, and mice genetically deficient in neurokinin-1 receptors are far less susceptible to the effects of toxin A than are normal mice (CASTAGLIOULO et al. 1994, 1997, 1998b; MANYTH et al. 1996a; POTHOULAKIS et al. 1998). Thus, toxin A appears to cause its enterotoxic effects partially by causing the release of neurohormonal substances that in turn alter secretion (MANYTH et al. 1996b). The action of cholera toxin is now believed to involve the enteric nervous system as well (JODAL and LUNDERGREN 1995).

2. *C. Sordellii* and *C. Novyi* Toxins

The biological properties of *C. sordellii* LT and HT are similar to those of *C. difficile* toxins B and A, respectively (POPOFF 1986; MARTINEZ and WILKINS 1988, 1992). Although a potent cytotoxin, LT is approximately 1000-fold less cytotoxic than toxin B. Interestingly, however, it is about tenfold more lethal for mice than toxins A or B when injected intraperitoneally. Like toxin B, LT is not an effective enterotoxin. HT and toxin A are remarkably similar. They have similar minimal cytotoxic and lethal doses, and each toxin is enterotoxic in amounts of 1–2 mg. Moreover, they share more extensive immunological cross-reactivities than do LT and toxin B. Thus, HT and toxin A appear to have diverged less than LT and toxin B.

Table 1. Molecular and biological properties of large clostridial toxins

	C. difficile toxin		*C. sordellii* toxin		*C. novyi* α-toxin
Property	A	B	HT	LT	
M_r (Da)	308000	270000	300000	270000	250000
pI	5.6	4.2	6.1	4.5	5.8
Cytotoxicity[a]	10 ng	1 pg	15 ng	1.6 ng	100 pg
Lethal dose[b] (ng)	50	50	75	5	5
Enterotoxicity[c]	1 m	–[d]	2 m	–	–
Vascular permeability	+	+	+	+	+
Hemagglutination[e]	+	–	+	–	–
Glucosyltransferase	+	+	+	+	+
Substrate	Rho, Rac, Cdc42	Rho, Rac, Cdc42	Rho, Rac, Cdc42	Rac, Cdc42, Ras, Rap, Ral	Rho, Rac, Cdc42
Co-substrate	UDP–Glc	UDP–Glc	UDP–Glc	UDP–Glc	UDP–GlcNAc

Glc, glucose; *GlcNAc*, *N*-acetylglucosamine; *HT*, hemorrhagic toxin; *LT*, lethal toxin; M_r, molecular weight; *UDP*, uridine diphosphate.
[a] Minimum dose (per tissue-culture well in a 96-well plate) causing over 50% rounding of Chinese hamster ovary K1 cells.
[b] Minimum lethal dose per mouse, determined by intraperitoneal injection into Balb/c mice.
[c] Minimum enterotoxic dose, determined in rabbit ileal loop assay.
[d] Toxin B from some atypical strains (8864) is enterotoxic.
[e] Agglutination of rabbit erythrocytes at 4°C.

C. novyi α-toxin also has biological properties that are similar to those of toxins A and B (Ball et al. 1993; Bette et al. 1989, 1990, 1991; Oksche et al. 1992). Its cytotoxic activity approaches that of toxin B, with a minimum cytotoxic dose of 100 pg, whereas its lethal dose approaches that of LT, with a minimum mouse lethal dose of 5 ng. α-Toxin does not have hemagglutinating activity, nor does it elicit a fluid response in ligated-loop assays. Due to its large size and similar biological activity, however, Bette et al. suggested that α-toxin may represent another member of the large clostridial toxins, which cause cell rounding (Bette et al. 1991). Cloning and sequencing of the α-toxin gene, along with studies showing that it glucosylates Rho proteins, established that it should indeed be grouped with the other large clostridial toxins. The molecular and biological properties of the large clostridial toxins are presented in Table 1.

3. Receptors

Toxins A and B enter the cell primarily by receptor-mediated endocytosis, as indicated by the fact that they bind to the cell surface, cluster in coated pits,

and are inhibited by agents that block endocytosis (Florin et al. 1983; Henriques et al. 1987). Both toxins contain a large series of repeating units at their *C*-end. In the case of toxin A, the repeating units form the portion of the toxin that binds to specific cell-surface carbohydrate receptors (Price et al. 1987; Pothoulakis et al. 1996). The repeating units of toxin A form a multivalent lectin that agglutinates rabbit erythrocytes. Its specificity for rabbit erythrocytes, which contain the carbohydrate galactose (Gal)-α1–3Gal-β1–4*N*-acetylglucosamine (GlcNAc), led to the discovery of the carbohydrate portion of toxin-A receptors (Krivan et al. 1986). We found that toxin A binds to hamster intestinal brush-border cells, which also contain Gal-α1–3Gal-β1–4GlcNAc. This trisaccharide is not expressed by normal human cells and, therefore, cannot be the receptor for toxin A in the human intestine.

Toxin A also binds to human carbohydrate antigens I, X, and Y, all of which have a conformationally similar type-2 core structure that is conformationally similar to Gal-α1–3Gal-β1–4GlcNAc (Tucker and Wilkins 1991; Smith et al. 1997). These antigens are all expressed on the surface of the human intestinal epithelium and may function as receptors for toxin A. Interestingly, the X antigen is also present in high amounts on the surfaces of human neutrophils and other leukocytes, indicating that toxin A may also target these cells. This may be important, since the inflammatory response plays a critical role in the pathogenesis of *C. difficile* infections. Glycoproteins from human intestinal epithelial cells, which bind toxin A, have not been identified. A 163-kDa glycoprotein receptor for toxin A in hamsters, however, has been purified from brush-border cells from the small intestines of infant hamsters (Rolfe and Song 1993). Binding was inhibited by lectins specific for Gal and GlcN-Ac and by non-immunoglobulin components of human milk (Rolfe and Song 1995). Toxin A does not bind to rabbit erythrocytes at 37°C, probably due to the low density of receptors on the red cell surface. In fact, the affinity of the repeating units for a single carbohydrate receptor appears to be low at 37°C. Effective binding at physiological temperatures, therefore, may require a high density of receptors. In this manner, toxin A may preferentially target specific cell lines, such as intestinal epithelial cells and leukocytes, which contain a high density of receptors. Receptors for toxin B and the other large clostridial toxins have not been identified.

C. Mechanism of Action

The similarity of toxins A and B, both structurally and in their effect on cells, suggested that they might have a similar mechanism of action. Although toxin B is significantly more potent as a cytotoxin, the effects of each toxin on tissue-culture cells are qualitatively very similar. Cells treated with the toxins exhibit a retraction of cell processes and rounding of the cell body (Ottlinger and Lin 1988; Thelestam and Bronnegard 1980; Fiorentini et al. 1990). The cytopathic effect results from disassembly of filamentous actin accompanied by a

decrease in F-actin and an increase in G-actin prior to the onset of cell rounding. The fact that the toxins were active in very small amounts supported the idea that toxicity is dependent on enzymatic activity within the target cell. Since the toxins cause cell rounding by disruption of the actin cytoskeleton, proteins regulating this process were likely targets of the toxins.

In this regard, the Rho/Rac family of low-molecular-weight guanosine triphosphate (GTP)-binding proteins, seemed ideal candidates for modification by the toxins. Some bacteria, including *Clostridium* sp., *Bacillus cereus*, and *Staphylococcus aureus*, produce ADP–ribosyltransferases specific for Rho (Weigers et al. 1991; Just et al. 1992a, 1992b; Aktories 1997). This Ras-related family of proteins, which includes RhoA, RhoB, RhoC, Rac 1, Rac 2, and CDC42, is involved in the regulation of actin-microfilament assembly (Hall 1998; Makay and Hall 1998). In 1994 and 1995, two research groups independently reported that toxins A and B do, in fact, modify Rho proteins (Just et al. 1994, 1995a; Dillon et al. 1995). Interestingly, the activities of toxins A and B were not due to ADP–ribosylation of Rho. Rather, Just et al. determined that toxins A and B covalently modify Rho by a novel mechanism that requires uridine diphosphate glucose (UDP; Glc) (Just et al. 1995b, 1995c). Each of the toxins is a glucosyltransferase that transfers a Glc from UDP–Glc to Rho. There are differences in the enzymatic potencies of toxins A and B, which may explain, at least in part, why toxin B is a more potent cytotoxin (Chaves-Olarte et al. 1997; Ciesla et al. 1998).

The similarity of toxins A and B with LT, HT, and α-toxin supported the idea that they would have similar enzymatic activity. Indeed, recent studies have shown that all of the large clostridial toxins modify the Rho/Ras subfamily of proteins by glucosyltransferase activity (Von Eichel-Streiber et al. 1996; Aktories et al. 1997). LT and α-toxin, however, exhibit interesting differences in substrate and co-substrate specificity when compared with *C. difficile* toxins (Genth et al. 1996; Hofmann et al. 1996; Just et al. 1996; Popoff et al. 1996; Selzer et al. 1996; Von Eichel-Streiber et al. 1996; Aktories 1997). The substrate specificity of LT is extended to the Ras subfamily of proteins. α-Toxin from *C. novyi*, however, modifies the same Rho subfamily members as toxins A and B but uses UDP–GlcNAc, rather than UDP–Glc, as a co-substrate. A detailed discussion of the mechanism by which the large clostridial toxins modify Rho is presented in Chap. 14.

D. Molecular Genetics of the Toxins

I. *C. Difficile* Toxin A and B Genes

Cloning and expression of a 0.3-kb fragment of the toxin A gene from *C. difficile* in a λgt11 library was reported in 1987 (Muldrow et al. 1987). Wren et al. also reported λgt11 clones containing a toxin-A gene fragment (Wren et al. 1989) The clone expressed a 235-kDa protein that caused elongation of

Chinese-hamster-ovary cells. The clones were unstable in both cases. Von Eichel-Streiber et al. reported cloning of overlapping fragments of the toxin-A gene (Von Eichel-Streiber et al. 1988). We reported cloning of the carbohydrate-binding portion of toxin A in pBR322 (Price et al. 1987). The cloned, 4.7-kb PstI restriction fragment expressed a protein that reacted with affinity-purified monospecific antibodies against toxin A and agglutinated rabbit erythrocytes. Cytotoxic activity, however, was not observed. The clone (designated pCD11) was used as the initial probe for chromosomal walking to clone-flanking regions of the *C. difficile* VPI 10463 chromosome (Dove et al. 1990; Johnson et al. 1990) As it turned out, the toxin A and B genes were located in close proximity, and this work led to the cloning and sequencing of both toxins.

Sequencing of the toxin-A gene by Dove et al. showed that the structural gene is 8130 nucleotides (nt) and encodes a 308-kDa protein which is 2710 amino acids in length, making it the largest known single-polypeptide bacterial toxin (Dove et al. 1990). The entire toxin-A gene was reconstructed from cloned fragments of the toxin-A gene and expressed in *Escherichia coli.* Lysates of recombinant *E. coli* expressing the toxin-A gene contained the cytotoxic activity, enterotoxic activity, and lethal properties of the toxin (Phelps et al. 1991). Moreover, antibodies for toxin A inhibited the activity of the lysates. This work firmly established that the toxic activity is attributable solely to the large, single-polypeptide protein produced by the toxin-A gene.

Chromosomal-walking experiments used for cloning the toxin-A gene led directly to cloning of the toxin-B gene (Johnson et al. 1990). The 3′ end of the toxin-B gene, as it turned out, is located 1350 bp upstream of the toxin-A initiation codon. Clone pCD19, which contains the 5′-end of the toxin-A gene and a small open reading frame (ORF), was found to contain 1.2 kb of DNA which, when subcloned, expressed a non-toxic peptide that reacted with toxin-B antibodies. The rest of the toxin-B gene was isolated on a 6.8-kb fragment that overlapped the pCD19 clone. The full-length toxin-B gene was reconstructed from the two overlapping fragments. The protein expressed by the complete gene reacted with toxin-B antisera and was cytotoxic and lethal. One surprising finding of this study was that the cloned toxin-B gene was expressed quite well in *E. coli* under control of its own promoter. This made it possible to proceed with mutagenesis studies on the toxin-B gene in *E. coli* (Barroso et al. 1994). Sequencing of the toxin-B gene by Barroso et al. revealed a structural gene 7098 nucleotides in length with a deduced amino acid sequence 2366 amino acids (M_r = 270 000 Da) in length (Barroso et al. 1990). Again, the gene sequence confirmed earlier findings that toxin B, like toxin A, is an unusually large protein expressed as a single polypeptide. The toxin-A and -B genes have also been cloned from VPI 10463 by Von Eichel-Steiber et al. (Von Eichel-Streiber et al. 1988, 1992a; Von Eichel-Streiber 1995). The sequencing data is essentially identical to that reported by our research group (Barroso et al. 1990; Dove et al. 1990).

II. *C. Difficile* Toxigenic Element

It has emerged, in recent years, that bacterial virulence factors are often obtained in clusters by horizontal gene transfer. These genetic "blocks" or "cassettes" are referred to as pathogenicity islands (Groisman and Ochman 1996; Hacker et al. 1997). In 1995, Hammond et al. described the boundaries of a genetic element containing toxins A and B in *C. difficile* VPI 10463 (Hammond and Johnson 1995). The gene block, which they termed the toxigenic element, is comprised of 19.6kb (Fig. 1). It contains three other small ORFs, termed *txe1* (also *txeR*), *txe2*, and *txe3*. Hundsberger et al. later also described the same element and referred to it as the pathogenicity locus, with the genes termed *tcdA-E* (Hundsberger et al. 1997). Two of the smaller ORFs, *txeR* and *txe2,* are transcribed in the same direction as the toxin gene (Hammond et al. 1996; Hundsberger et al. 1997). Transcript levels peak during the stationary phase, along with those of toxins A and B. Positive regulation of the toxin genes is controlled by *txeR* (see below). The protein encoded by *txe2* contains a potential lipoprotein signal sequence at its N-terminus. Its function is unknown. The additional ORF, *txe3*, is transcribed in the opposite direction. Its messenger RNA (mRNA) transcript peaks during exponential growth and is not present during the stationary phase (Hundsberger et al. 1997). It has been proposed that it may play a role in negative regulation of the toxin genes, although this remains to be determined.

Most pathogenicity islands are comprised of a large region of DNA and encode many virulence-related proteins. Due to its small size, the toxigenic element may be more properly termed a pathogenicity islet. Non-toxigenic strains of *C. difficile* contain a 127-bp sequence at this locus, which is not found in toxigenic strains. This sequence, which contains inverted repeats with predicted secondary structure, may represent a target for insertion of the toxigenic element. We recently described a similar pathogenicity islet in *B. fragilis*, which contains the genes for a proteolytic enterotoxin termed fragilysin and another very similar metalloprotease (Moncrief et al. 1998). Non-toxigenic strains of *B. fragilis* also contain a 17-bp sequence, which may serve as a target for integration of the fragilysin pathogenicity islet.

III. Atypical Strains of *C. Difficile*

Toxigenic strains of *C. difficile* almost invariably produce both toxins. In 1991, however, Torres identified a strain (8864) which produces only toxin B (Torres

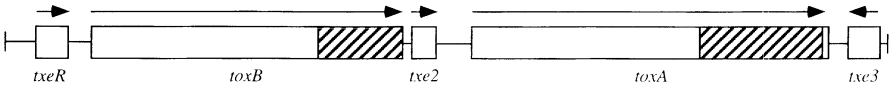

Fig. 1. Toxigenic element of *Clostridium difficile* VPI 10463. *Striped boxes* represent the repeating units of the toxins. *Arrows* indicate the direction of transcription. The protein encoded by *txeR* positively regulates expression of both toxins

1991). Interestingly, toxin B produced by 8864 was found to be a more potent toxin than toxin B produced by VPI 10463. Moreover, 8864 is virulent in hamsters, and toxin B isolated from this strain causes fluid secretion in rabbit intestinal loops (Borrielo et al. 1992; Lyerly et al. 1992). These findings suggested that atypical strains of *C. difficile* might cause disease despite the fact that they do not produce toxin A.

So far, two different types of toxin-B-positive/toxin-A-negative strains have been identified (Depitre et al. 1993; Rupkin et al. 1997; Soehn et al. 1998). One group is represented by 8864. These strains have a large deletion (5.6 kb) in the toxin-A gene. Additionally, they have a DNA insert of about 1.1 kb between *txe2* and the beginning of the toxin-A gene. The other group is the serotype-F strains (strain 1470). They have been isolated from infants and, so far, do not appear to cause disease in humans or animals. Polymerase-chain-reaction analysis indicates that they are deleted for only a small portion (~1.7 kb) of the toxin-A gene within the repeating units. This portion of the repeats contains the epitopes for toxin-A monoclonal antibodies used in toxin-A enzyme-linked immunosorbent assays (ELISAs), explaining why these strains are negative in immunoassays for toxin A (Lyerly et al. 1983; Frey and Wilkins 1992). This region is also responsible for toxin-A binding to intestinal cells, offering a reason for the lack of biologically active toxin A. Recently a *C. difficile* strain producing only toxin A has been identified in a patient with AAD (Cohen et al. 1998). Whether or not atypical strains of *C. difficile* are causing disease in humans is an important question. If so, there are important issues to address regarding diagnosis, treatment and prevention strategies.

IV. *C. Sordellii* and *C. Novyi* Genes

The gene for *C. sordellii* LT has recently been cloned and sequenced (Green et al. 1995). The structural gene is similar in size to the *C. difficile* toxin-B gene, with a corresponding M_r of 270 000 Da. It is 7095 nucleotides in length and encodes a 2364-amino-acid toxin. The deduced amino acid sequence of LT shares 76% identity with toxin B and 47% identity with toxin A, confirming that it is more closely related to toxin B. HT probably shares close sequence identity with toxin A; however, the HT gene has not been cloned and sequenced. Recently, an enterotoxin-negative, cytotoxin-positive atypical strain of *C. sordellii* has been isolated (Green et al. 1996).

The α-toxin gene has been cloned and sequenced (Hofmann et al. 1995). The gene is 6534 nucleotides in length and encodes a 2178-amino-acid protein of M_r 250,000 Da. The deduced amino acid sequence shows 48% identity with toxins A and B. Moreover, like toxins A and B, it contains a series of repeat motifs at the C-terminal portion of the molecule and a central hydrophobic region with potential membrane-spanning segments. The *C. novyi* α-toxin gene is carried as part of a lysogenic phage (Eklund et al. 1974, 1976; Schallehn et al. 1980). Toxigenic strains cured of the phage no longer produce α-toxin, and non-toxigenic strains are converted to toxin-producing strains by phage

infection. Phage transduction has not been described for the other large clostridial toxins.

V. Sequence Identity and Conserved Features of the Toxins

A comparative sequence analysis of the toxin-A and -B genes confirmed their relatedness at the structural level (Von Eichel-Streiber et al. 1992a; Von Eichel-Streiber 1995). Alignment of the amino acid sequences showed extensive sequence identity. The toxins share 49% amino acid identity and are 63% similar if conservative substitutions are considered. The sequence identity is striking in some areas. The extensive sequence identity and similar structure and enzymatic activity of the toxins, combined with the fact that the genes are in close proximity on the chromosome, suggest that the genes resulted from gene duplication (Von Eichel-Streiber 1995). LT and α-toxin also have considerable sequence identity and contain the conserved features shared by toxins A and B shown in Fig. 2.

1. N-Terminal Glucosyltransferase Domain

Protein toxins typically are composed of an N-terminal enzymatic domain and a C-terminal receptor-binding domain. It was assumed, therefore, that the enzymatic activity of the large clostridial toxins would be found in the N-terminal portions of the toxins. Because of the unusually large nature of the

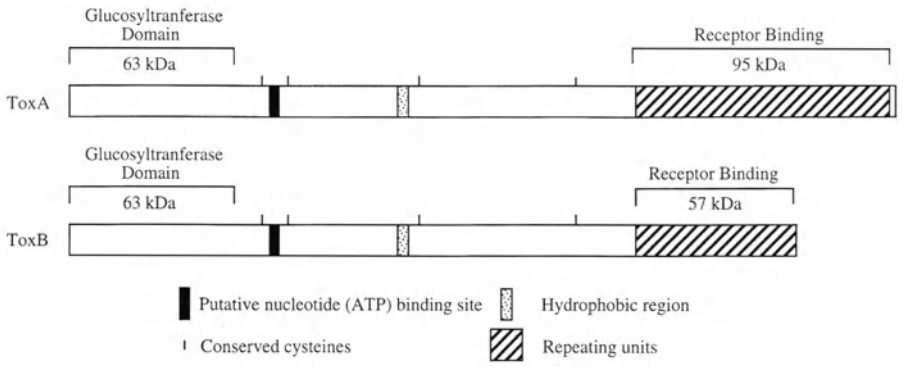

Fig. 2. Conserved features of *Clostridium difficile* toxins A and B. Toxin A contains 2710 amino acids and has a molecular weight (M_r) of 308000 Da. Toxin B contains 2366 amino acids and has a M_r of 270000 Da. The following are numbers of the amino acids for structural features of each toxin. Glucosyltransferase domain: toxin A = 1–546 (assumed); toxin B = 1–546. Putative nucleotide-binding site: toxin A = 653–686; toxin B = 651–684. Hydrophobic region: toxin A = 1064–1114; toxin B = 1062–1112. Conserved cysteines: toxin A = 597, 700, 1169, 1623; toxin B = 595, 698, 1167, 1625. Repeating units: toxin A = 1849–2680; toxin B = 1851–2366. Toxin A contains a hydrophobic stretch of amino acids (2681–2710) at its carboxyl end, represented by *a small open box*. The other large clostridial toxins contain similar structural features

toxins, this meant that, upstream of the toxins' binding domains, there was approximately 200 kDa in which the enzymatic activity might be contained.

A small hydrophobic region is located approximately in the center of this 200-kDa region. Thus, it was speculated that the enzymatic activity might be located in the N-terminal third of the toxins. The first evidence for this came from our studies of a deletion mutant of toxin B (Barroso et al. 1994). This mutant (toxB-H:M) lacked a large part of the middle region of the toxins but contained the N-terminal third of toxin B, fused in-frame with the repeating units. Activity of the mutant toxin was dramatically reduced. However, it did retain some activity, suggesting that enzymatic activity was indeed contained in the N-terminal third of the holotoxin. Confirmation of this came from a detailed analysis of the in vitro glucosyltransferase activity of various recombinant fragments from within this region. In our earlier study, site-directed mutagenesis of His653 to Glu [in a putative nucleotide-binding site (Fig. 2) located within the N-terminal domain] suggested that it might be the center of enzymatic activity. This mutant toxin B lost 99.9% of its cytotoxic activity. Interestingly, however, the enzymatic activity was found by Hofmann et al. to reside entirely within the first 546 amino acids (63 kDa) of toxin B (Hofmann et al. 1997). This region does not contain the putative nucleotide-binding site. The 63-kDa fragment of toxin B had as much glucosyltransferase activity as the holotoxin. Furthermore, the recombinant protein caused cell rounding when microinjected into NIH-3T3 cells. These findings were confirmed and extended in additional studies (Wagenknecht-Wiesner et al. 1997). The enzymatic activity of the other large clostridial toxins is likely contained within this relatively small portion of the holotoxins. In fact, the enzymatic domain of *C. sordellii* LT has recently been shown to reside within the first 546 N-terminal amino acids (Hofmann et al. 1998). The precise enzymatic site and critical residues involved in catalysis by the toxins have not been determined.

2. Repeating Units

One of the most striking features of the toxins is the presence of repeating units encoded by nearly one-third of the genes at their 3′ end. In toxin A, for example, this region contains a series of 38 contiguous repeating units composed of 831 amino acids. Based on their lengths and low levels of sequence similarity, the repeating units were grouped (by Dove et al.) into classes I and II. Toxin A contains seven of the class-I repeating units, each of which is 30 amino acids in length, and 31 class-II repeats, which contain 20 or 21 amino acids each. The class-II repeats were subdivided into four groups: A, B, C, and D. They contain at least two epitopes for the monoclonal antibody PCG-4 and are the immunodominant portion of the molecule (Frey et al. 1992). The other large clostridial toxins contain similar repeating units at their C-ends. These repeating units share homology with the carbohydrate-binding region of streptococcal glycosyltransferases (GTFs; Von Eichel-Streiber et al. 1990, 1992a).

The GTFs and repeating units of toxins A and B are rich in aromatic amino acids and, with rare exceptions, each unit contains the sequence YYF. All the repeating units contain YF, which has been used to align the sequences for classification into subgroups based on additional sequence similarities (Dove et al. 1990; Von Eichel-Streiber et al. 1990). It has been proposed that the repeats have a modular design in which the aromatic amino acids function in primary protein–carbohydrate interactions (Von Eichel-Streiber et al. 1992b). The affinity for binding is amplified by the repetition of the sequence, and binding specificity is determined by a second, as yet undefined, characteristic of the arrangement and sequence of the repetitive structure. In this manner, the proteins have evolved an ability to bind different carbohydrate structures based on a similar fundamental unit.

3. Additional Conserved Features

Toxins A and B share several additional, distinct structural features, including four conserved cysteines, a potential nucleotide-binding site, and a central hydrophobic region (Fig. 2). The segment containing a large number of hydrophobic amino acids is located in the center of the toxins. Some other bacterial toxins have centrally located hydrophobic regions that are thought to function as membrane-spanning regions necessary for toxin transport. Whether or not the hydrophobic centers of toxins A and B have a similar function remains to be determined. Additional clusters with exceptional sequence identity are also present within the region between the hydrophobic area and the beginning of the repeating units (Von Eichel-Streiber 1995). The function of the four conserved cysteines is not known. Reducing agents do not affect the toxins, suggesting that the cysteines are not involved in formation of disulfide bridges critical to toxin structure. Instead the cysteine residues may function as free sulfhydryls involved in ligand binding. Alternatively, the conserved cysteines may be strategically located near processing points essential for toxin activation inside the cell.

We developed a series of deletion and site-directed mutants of the toxin-B gene altered in these conserved features and studied the effects on cytotoxicity (Barroso et al. 1994; Moncrief et al. 1997b). Three deletion mutants of the C-terminal end were developed to investigate the role of the repeating units and the fourth cysteine. The toxin-B gene was deleted from the 3′ end by exonuclease digestions. Removal of the repeating units of toxin B resulted in a tenfold reduction in activity. Surprisingly, however, the mutant toxin still had significant activity, since the recombinant lysate had a cytotoxicity titer of 10^5, compared with 10^6 for the unaltered toxin. Removal of a further 125 amino acids decreased the cytotoxicity another tenfold; however, a significant amount of activity (10^4) still remained. In contrast, deletion of an additional 141 amino acid region containing the fourth conserved cysteine resulted in complete inactivation of the toxin. Substitution of the fourth cysteine (Cys1625) with serine also resulted in a tenfold decrease in activity. When serine was substituted for

the second conserved cysteine (Cys698), located downstream of the proposed nucleotide-binding site, a tenfold decrease in activity was observed. As discussed above (Sect. D.V.1), substitution of a histidine residue (His653) in the putative nucleotide-binding site eliminated 99.9% of the cytotoxic activity. Taken together, these results illustrate the importance of the large central domain of the toxins, despite the fact that it does not exhibit glucosyltransferase activity.

VI. Gene Transfer in *C. Difficile*

The lack of a system for gene transfer in *C. difficile* has hampered the analysis of its virulence factors, including the toxins. In this regard, MULLANY et al. have reported some recent success on conjugative gene transfer in *C. difficile* (MULLANY et al. 1990, 1991, 1994). Site-specific integration into the *C. difficile* chromosome was accomplished using pCI195, a pBR322-based vector containing a 4.2-kb region of the conjugative transposon Tn919. Conjugative transfer required a *Bacillus subtilis* strain that contained the related transposon Tn916ΔE as an intermediate host. The transposon::plasmid structure was transferable from *B. subtilis* to *C. difficile* by filter mating at a frequency of 10^{-8}. They further demonstrated that pCI195 could be used for gene cloning into *C. difficile* by transfer of a 1.1-kb fragment of the toxin-B gene into a nontoxigenic strain of *C. difficile*. The transposon::plasmid structure entered the genome at a specific site, as occurs in *B. subtilis*. The ability to mobilize genes into *C. difficile* means it may now be possible to study gene function in *C. difficile* directly. Unfortunately, the methods developed by MULLANY et al. are limited to the introduction of small segments of DNA as part of a conjugative transposon. The large size of the toxin genes, therefore, prohibits their study using this system of conjugal gene transfer. Direct transformation of *C. difficile* with plasmid DNA has not been demonstrated. *C. difficile* strains, however, often contain cryptic plasmids; these could provide a basis for developing functional cloning vectors for *C. difficile*. Genetic transformation of *C. sordellii* or *C. novyi* has not been reported.

E. Regulation of *C. Difficile* Toxins

It has been known for years that *C. difficile* produces far more toxin when grown under conditions that slow the growth of the organism. Conditions that limit nutrient availability, such as growth in dialysis sacs, leads to very high levels of toxins A and B. The botulinum and tetanus neurotoxins are also produced in higher amounts when grown in dialysis-sac cultures, suggesting similar regulatory control. Limitation of biotin in defined media increases the production of *C. difficile* toxins (YAMAKAWA et al. 1996). Rapid growth in rich media, however, essentially halts toxin production. Toxin expression is also repressed by Glc (BRUNO and SONENSHEIN 1998).

Putative promoters of the toxin-A and -B genes were proposed by Von Eichel-Streiber et al. (1995). The proposed promoters have unusual distances between the transcriptional start site and the ATG start codons (169 nucleotides for toxin A and 239 nucleotides for toxin B). Similar spacing is found in the promoter regions of tetanus- and botulinum-toxin genes and the ultraviolet (UV)-light-inducible promoter P1 of the *C. perfringens*. Recent studies verified that these promoters, which share sequence identity, are indeed functional promoters of the toxin-A and -B genes (Hammond et al. 1996; Hundsberger et al. 1997; Bruno and Sonenshein 1998; Song and Faust 1998).

The *txeR* gene, located immediately upstream of the toxin B gene, encodes a small (22-kDa) basic protein, which is rich in lysine residues at its C-terminus. Furthermore, it contains a helix–turn–helix motif with sequence identity to those of DNA-binding proteins. TxeR also shares 22% identity with UviA, the putative regulator of the UV-inducible promoter P1. These characteristics suggested to us that it may play a role in regulation of toxin-gene expression. We used a DNA fragment encoding the toxin-A repeating units (ARU) as a reporter gene to determine if *txeR* regulates expression from the toxin-A and -B promoters in *E. coli* (Moncrief et al. 1997a). Expression of ARU was measured using ELISA. When *txeR* was supplied in *trans* expression from the toxin-B promoter–ARU increased over 800-fold. In similar experiments, expression from the toxin-A promoter–ARU fusion was increased over 500-fold in the presence of *txeR*. Thus, *txeR* encodes a *trans*-acting regulatory protein that positively regulates expression from both toxin promoters. Previously, based on detailed analysis of toxin-gene transcripts, Hammond et al. suggested that the toxins are transcribed as part of a very large (17.5-kb), polycistronic mRNA and that smaller mRNAs, which were also detected, may result from post-translational processing (Hammond et al. 1996). Our studies with *txeR* in *E. coli*, however, suggest that the majority of toxin-gene transcription takes place from the individual toxin-gene promoters, and that high-level expression during stationary-phase growth is dependent on the *txeR*-gene product. The large transcript observed may be the result of incomplete transcriptional termination during high-level expression.

TxeR also contains significant homology to ORFs located near the tetanus- and botulinum-toxin genes (Eisel et al. 1986; Fairweather and Lyness 1986; Hauser et al. 1994; Henderson et al. 1996). These proteins, which also contain a helix–turn–helix DNA-binding motif, may positively regulate the tetanus- and botulinum-toxin genes. Furthermore, TxeR contains some similarity to particular σ factors known as the ECF (extracellular function) family (A.L. Sonenshein, personal communication). In gram-positive organisms, these σ factors act as general stress–response sigma factors (Missiakas and Raina 1998). TxeR, therefore, may not function as a typical transcriptional activator-response regulator. Rather, TxeR and its homologues in *C. botulinum* and *C. tetani* may be σ factors that turn on toxin production in response to stress (limited nutrients). Expression of TxeR itself may be controlled by upstream events. In turn, high-level transcription form the toxin-gene pro-

moters is initiated when TxeR becomes available. In some cases, ECF σ factors have also been shown to be modulated by a membrane protein that has anti-σ activity. These membrane proteins are thought to serve as sensors and signaling molecules which allow adaptive response to specific environmental conditions. Due to their similarity to toxins A and B and their production at high levels in dialysis-sac cultures, the large toxins of *C. sordellii* and *C. novyi* may be controlled by regulatory genes similar to TxeR. Nothing is known, however, about any other genes that control the expression of the large clostridial toxins.

F. Conclusions

A great deal remains to be learned about these toxins. Approximately the final third of each toxin appears to function in binding. The enzymatic site responsible for cytotoxicity is contained within the first 63 kDa, which comprises less than a fourth of each molecule. This leaves a very large part of the toxins for which the function is unknown. This region between the end of the enzymatic domain and the beginning of the repeating units has a mass of approximately 150 000 Da. This is larger than most bacterial proteins, including most toxins.

Recent breakthroughs have clearly established that the large clostridial toxins covalently modify low-M_r GTP-binding proteins that regulate the actin microfilaments. It is now appreciated that these GTPases are central players in the regulation of many cellular functions, including cell division, motility, and intercellular communication (Hall 1998; Makay and Hall 1998). A number of other bacterial enzymes modify the Rho/Rac family of proteins by ADP–ribosylation (Aktories 1997; Sehr et al. 1998). Most of these enzymes, however, do not contain receptor-binding domains and are, therefore, less useful than the large toxins produced by *C. difficile*, *C. sordellii*, and *C. novyi* as tools for studying cell biology. Indeed, in the few years since the mechanism of action of the large clostridial toxins became known, numerous studies of cell function, using toxins A and B as molecular tools, have been reported. Undoubtedly, further studies on the molecular biology of the large clostridial toxins will provide further important insights into cellular function.

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CHAPTER 16

The Cytotoxic Necrotizing Factor 1 from *Escherichia Coli*

P. Boquet and C. Fiorentini

A. Introduction

Escherichia coli is among the most important causes of intestinal infections. These bacteria, which are normal flora of the intestine, become highly pathogenic upon acquisition of genes coding for virulence factors. Apart from those strains acting in the intestine, there are distinct *E. coli* strains that have the ability to cause a wide variety of extra-intestinal diseases, including septicemia and meningitis in neonates and urinary infections in children and adults. In the case of urinary-tract infections, *E. coli* strains often express exotoxins, such as hemolysin and cytotoxic necrotizing factor 1 (CNF1), together with colonization factors, such as Pap or Afa adhesins (Caprioli et al. 1987, 1989; Blanco et al. 1992; Falbo et al. 1992; Blum et al. 1995; Swenson et al. 1996). Genes coding for these virulence factors are linked (Blum et al. 1995; Swenson et al. 1996) within regions of chromosomal DNA which have been called pathogenicity islands (PAIs; Groisman and Ochman 1996; Hacker et al. 1997) on the chromosome of uropathogenic *E. coli* J96 strains. CNF1 is a relatively recently discovered 110-kDa *E. coli* protein toxin (Caprioli et al. 1983) which induces, in cultured cells, an impressive reorganization of actin microfilaments (Fiorentini et al. 1988) via activation of the 21-kDa Rho–guanosine triphosphate (GTP)-binding protein (Fiorentini et al. 1994, 1995, 1997a; Oswald et al. 1994), which takes place via a new catalytic activity for bacterial toxin (Flatau et al. 1997; Schmidt et al. 1997). Although knowledge of the biological and molecular properties of CNF1 has accumulated over the last 10 years, there is only indirect evidence to substantiate the role of this toxin as a bona fide virulence factor. The main difficulty in implicating CNF1 in the virulence of CNF1-producing *E. coli* is the fact that the toxin remains associated to the bacteria and is not detectably released in the culture medium. In the present review, we discuss our present knowledge of the genetics of CNF1, its impact in uropathogenic infections, its structure–function relationships and cellular effects and its possible role as a virulence factor.

B. The CNF1 Gene and the Prevalence of CNF1-Producing Strains among Uropathogenic *E. coli*

I. The CNF1 Gene

CNF1 is chromosomally encoded in uropathogenic *E. coli* by a single 3042-bp structural gene [Falbo et al. 1993; accession number (no.) X70670]. Some strains of enterotoxigenic *E. coli* found in animals produce CNF2, a toxin highly homologous (85.7% identical residues) to CNF1 (Oswald et al. 1994; accession no. U01097). The CNF2 gene is, however, encoded by a plasmid called "Vir" (De Rycke et al. 1990; Oswald and De Rycke et al. 1990). No classical signal sequence is found at the level of the CNF1 (or CNF2) terminal sequence. The GC% of the CNF1 (and CNF2; 35%) gene is lower than that of the *E. coli* genome (Falbo et al. 1993; Oswald et al. 1994). CNF1 belongs to a PAI (Blum et al. 1995; Swenson et al. 1996) that has been well studied in the uropathogenic *E. coli* strain J96 (Blum et al. 1995; Swenson et al. 1996). In the J96 strain, two pathogenicity islands have been mapped to the 64-min (PAI IV) and 94-min (PAI V) regions of *E. coli* K12. Only PAI V contains the CNF1 gene (Blum et al. 1995; Swenson et al. 1996). PAI V is a 110-kb DNA insert incorporated in the K12 chromosome at the phenylalanine transfer RNA gene *pheR* (Blum et al. 1995; Swenson et al. 1996). The PAI V is flanked, on each of its extremities, by an imperfect, 135-bp direct repeat, suggesting a homologous recombination process for insertion of the PAI. In the J96 strain, the gene of CNF1 is located between the hemolysin-α operon and the fimbriae adhesin protein Prs (Swenson 1996; Fig. 1). There are about 1 kb between the start codon of CNF1 and the stop codon of the *hlyC* gene (Falbo et al. 1993). The distance between the CNF1 stop codon and the first gene of the fimbriae adhesin operon is not yet known. All uropathogenic strains of *E. coli* expressing CNF1 studied so far contain the hemolysin-α gene. It has to be emphasized that, in the J96 strain, the *hlya* gene is contained in both PAIs (PAI IV and V; Blum et al. 1995; Swenson et al. 1996) but, as mentioned before, the CNF1 gene is only located in PAI V. In the J96 strain, the fimbriae gene associated with the *hly* operon and CNF1 in PAI V is that of the *pap*-like adhesin *prs* (Swenson et al. 1996).

The vast majority (76%) of uropathogenic *E. coli hly* and *cnf1*$^+$ are associated with *pap*/*prs* together with *S-fimbriae* (*sfa*) genes (Landraud and Boquet, submitted). In the same study, it has been shown that 40% of these *E. coli* had only the *sfa* adhesin gene (Landraud and Boquet, submitted). A minute proportion (12%) of uropathogenic *E. coli* harboring *hly* and *cnf1* genes contain only the *pap*/*prs*; also, 12% does not contain genes coding for adhesins (Landraud and Boquet, submitted). This demonstrates that the PAI containing the *cnf1* gene is strictly associated with the *hly* operon but might vary in terms of adhesins. We can make the working hypothesis that CNF1 and Hly α-hemolysin may act together in a mechanism of virulence that will give an advantage to the bacteria. This will be discussed later in this review. The

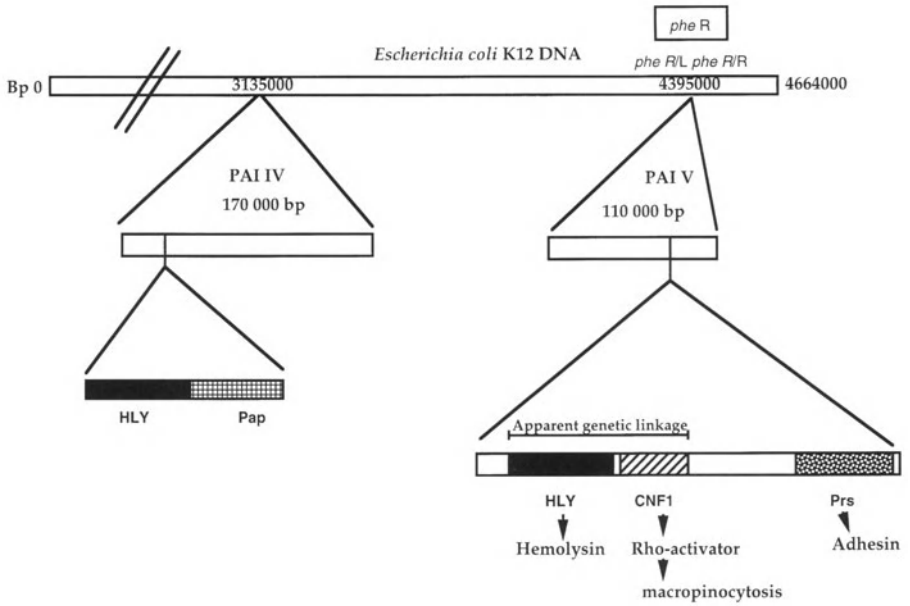

Fig. 1. The J96 pathogenicity island (*PAI*) V contains the cytotoxic necrotizing factor 1 gene. Schematic diagram of *Escherichia coli* J96 DNA chromosome, with positions of PAI IV and PAI V. Positions on *E. coli* chromosome of PAI IV and V are given as nucleotide base-pair designations

CNF1 protein does not contain an identifiable signal sequence and, therefore, it is not possible that this toxin uses a sec-dependent type-2 secretion mechanism. Furthermore, the *cnf1* gene does not belong to an operon like the *hly* operon, which codes for the hemolysin structure, for the secretion apparatus (type 1) of this pore-forming toxin and for the post-translational activation of the toxin by acylation (Koronakis and Hughes 1996). It remains to speculatively assign a mechanism of secretion comparable to those of virulence factors of *Shigella*, *Yersinia* or *Salmonella* (cell-contact-dependent secretion or type-3 secretion; Hueck 1998) to CNF1.

II. Prevalence of CNF1-Producing Strains among Uropathogenic *E. coli*

The prevalence of CNF1-producing *E. coli* among uropathogenic *E. coli* is now documented. According to Blanco and coworkers (1996), about one-third of uropathogenic *E. coli* produce CNF1. Recently, in a survey of 175 uropathogenic *E. coli* strains at the University Hospital of Nice, 33% of these strains were able to produce CNF1 (Landraud and Boquet, submitted), confirming the previous findings (Blanco et al. 1996). When the causative bacteria were separated on the basis of the urinary-infectious process (cystitis, acute

pyelonephritis or asymptomatic bacteriuria), exactly the same percentage of CNF1-producing *E. coli* was found in each group (Landraud and Boquet, submitted). However, since CNF1 is not present in all pathogenic strains and its secretion mechanism is not known, the role of this toxin as a bona fide virulence factor for uropathogenic *E. coli* is still questionable. Curiously, a gene having a homology with the *cnf1* gene has recently been found in the fish-associated, non-pathogenic, light-emitting bacteria *Vibrio fischeri* (Lin et al. 1998).

C. Production, Purification and Cellular effects of *E. coli* CNF1

I. Production and Purification of *E. coli* CNF1

CNF1 is a single-chain protein toxin (Falbo et al. 1993) with a predicted molecular mass of 1137 kDa (1014 amino acids). The toxin is produced at a very low level in wild-type pathogenic *E. coli* strains, such as BM2-1 (De Rycke et al. 1989), and this does not allow one to obtain a sufficient amount of proteins for toxin purification (De Rycke et al. 1989). Production and purification of CNF1 is usually obtained in our laboratories from *E. coli* strains (Epicurian Xl-1 blue or TG1) harboring the CNF1 gene and its promoter region (a minimum of 300 bp upstream of the ATG codon of CNF1) cloned in a multicopy plasmid. Freshly transformed bacteria grown in LB medium containing the selective antibiotic for plasmid maintenance are cultivated, with agitation, for 14 h at 37°C. The bacterial pellet resuspended in tris(hydroxymethyl)aminomethane (Tris)/HCl buffer (pH 7.5) is submitted to French-press disruption to release CNF1 from the bacterial cytoplasm. The toxin is concentrated by ammonium-sulfate precipitation (50% saturation) and, after dialysis against 50 mM Tris buffer (pH 7.5), is applied to a diethylaminoethyl (Pharmacia, Sweden) column and eluted as a sharp peak with 200 mM NaCl (Falzano et al. 1993). The toxin is then further purified by gel filtration and by fast protein liquid chromatography on a MonoQ (Pharmacia) column with a NaCl gradient (50–300 mM; Flatau et al. unpublished). Recombinant CNF1, fused with the glutathione-*S*-methyl transferase (GST; Schmidt et al. 1997) or made as a His-tagged fusion protein (Flatau et al. unpublished), is also used, although, in our studies, these recombinant proteins were usually obtained in small amounts Furthermore, to release native CNF1 from the fusion proteins, it is necessary to cleave the GST or His-tagged toxin with thrombin or factor X – a cumbersome technique for CNF1, with the further inconvenience that thrombin or factor X must be eliminated from the final preparation. The purified toxin is usually kept frozen in small aliquots at –80°C, where it can be stored several months without losing activity. Freezing and thawing CNF1 several times may reduce its cell activity.

II. Cellular Effects of *E. coli* CNF1

CNF1 was first described as a dermonecrotic toxin on injection into rabbit skin and as inducing multinucleation in cultured HeLa cells (Caprioli et al. 1984). However, the observation that CNF1 is able to induce the formation of thick actin stress fibers and membrane actin folding (Fiorentini et al. 1988) suggested that this toxin could operate via the regulation of the actin cytoskeleton (Fiorentini et al. 1988). The effects of CNF1 on cultured cells is indeed remarkable, and it contrasts with those of other cytoskeletal active toxins, such as *Clostridium botulinum* exoenzyme C3 (Chardin et al. 1989), *C. difficile* toxin B (Just et al. 1995) or *C. sordellii* lethal toxin (Just et al. 1996; Popoff et al. 1996), which cause an actin-filament-network breakdown. The induction of membrane actin folding and multinucleation by CNF1 are selectively observed in epithelial HEp-2 cells (Fig. 2B), whereas fibroblastoid cells, such as Vero or 3T3 mouse cells, are the best cell models for actin stress-fiber formation (Fig. 2D). The concentration of CNF1 in the cell-culture medium determinates the velocity at which the cytoskeletal effects occur. With very high concentrations of CNF1 (10^{-7}–10^{-8} M), membrane folding as fast as 40 min to 1 h is observed in HEp-2 cells. Formation of filopodia and lamellipodia can be observed upon treatment of HEp-2 cells with CNF1 (Fig. 2E). Vacuolation of HEp-2 cells, very different from that of the vacuolating toxin Vac A from *Helicobacter pylori* (Cover 1996), originates from cell-membrane folding and is probably due to macropinocytosis (Figs. 2F, 3A). With high concentrations of CNF1, thick stress fibers are also rapidly formed in Vero or mouse 3T3 fibroblasts. With very low amounts of CNF1 (10^{-11}–10^{-12} M), the appearance of the cytopathic effects is delayed for 10–24 h. After membrane actin-folding induction, CNF1 allows spreading of epithelial cells, such as HEp-2, and this is observed after 10–24 h. Some cell species, such as lymphocyte T immortalized cells (Jurkat), seem to be resistant to CNF1 (Boquet et al., unpublished). Polarized cells, such as the intestinal T84 (Hofman et al. 1998) and Caco2 cells (Gerhard et al. 1998), or dog kidney cells Madin-Darby canine kidney (MDCK; Fiorentini et al. unpublished) are fully sensitive to CNF1.

D. CNF1 Molecular Mechanism of Action

I. Intracellular Enzymatic Activity of CNF1

Growth factors, such as epithelial growth factor, platelet-derived growth factor and bombesin, and certain lipids, such as lysophosphatidic acid, can stimulate the reorganization of F-actin structures in cells through small, p21-Ras-like GTP-binding proteins of the Rho family (Tapon and Hall 1997; Hall 1998). Formation of stress fibers and focal contacts has been observed on stimulation of the Rho GTPase (Ridley and Hall 1992). Stimulation via Rac has

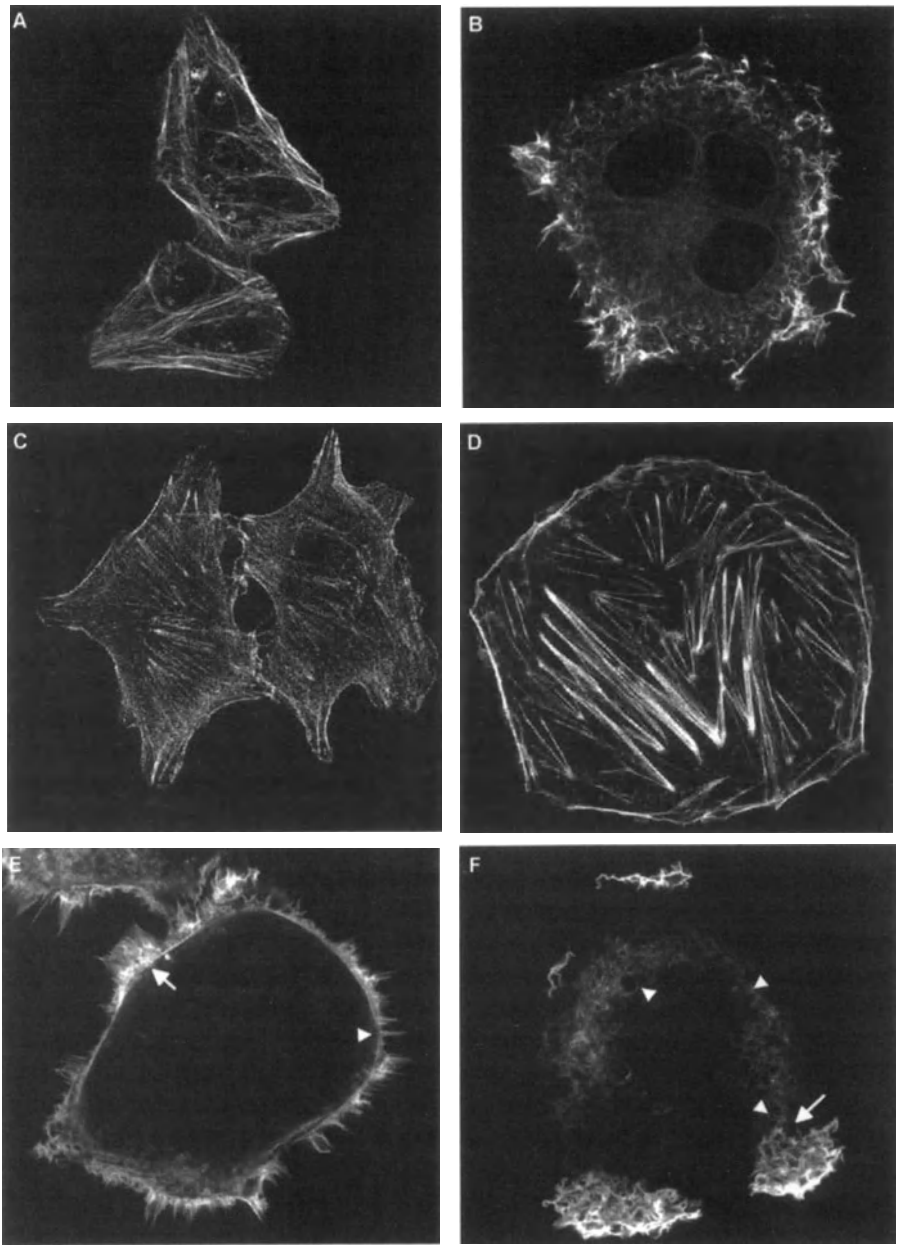

Fig. 2. Effects of cytotoxic necrotizing factor 1 (CNF1) on the actin cytoskeleton (stained by fluorescein isothiocyanate–phalloidin) on either HEp-2 or Vero cells. **A** Control Hep-2 cells. **B** HEp-2 cells treated with 10^{-10} M CNF1 for 12 h. **C** Control Vero cells. **D** Vero cells treated with 10^{-10} M CNF1 for 12 h. **E** HEp-2 cells treated with 10^{-8} M CNF1 for 4 h. *Arrow* shows a lamellipodia and *arrowhead* a filopodia. **F** HEp-2 cells treated with 10^{-10} M CNF1 for 4 h. *Arrow* shows the polarized membrane folding and *arrowheads* show vacuolar formations, which are more visible by phase contrast in Fig. 3A

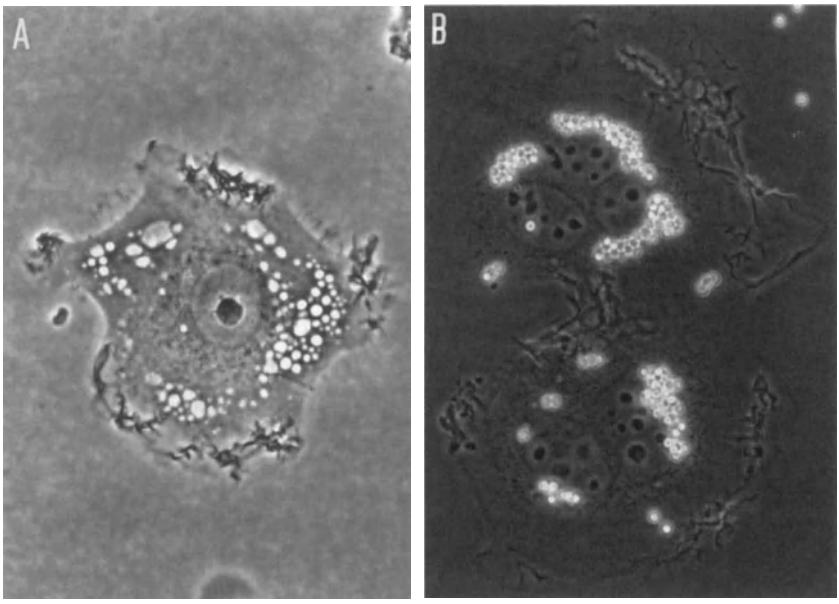

Fig. 3. Macropinocytosis induced by cytotoxic necrotizing factor 1 (CNF1). **A** Formation of vacuoles, induced by treating HEp-2 cells with 10^{-8}M CNF1 for 4h. **B** Macropinocytosis of latex beads by HEp-2 cells treated with CNF1

been shown to induce ruffling (Ridley et al. 1992), whereas triggering of Cdc42 was shown to induce formation of filopodia (Kozma et al. 1995; Nobes and Hall 1995). Rho regulates the actin cytoskeleton through three main mechanisms:

1. Bundling and contractility of actin filaments by inhibition – via phosphorylation by the Rho kinase (Rok or Rock; Ishizaki et al. 1996; Matsui et al. 1996) – of the myosin light-chain phosphatase (Kimura et al. 1996).
2. By formation of phosphatidyl-4-5-bisphosphate (PIP2) through activation of the phosphatidylserine-5P-kinase (Chong et al. 1996). PIP2 is thought to induce actin polymerization by uncapping barbed-end actin filaments (Hartwig et al. 1996; Schafer et al. 1996).
3. By activating proteins from the ezrin/radixin/moesin (ERM) family that make a link between the cell membrane and the actin cytoskeleton, thus promoting the formation of focal contact points (McKay et al. 1997).

CNFl induces actin-cytoskeleton reorganization in cells by permanently stimulating the Rho protein. The first hint of this activity was shown as follows: when the cytosol from HEp-2 cells previously incubated with CNF1 was adenosine diphosphate (ADP)–ribosylated with exoenzyme C3, the Rho protein showed a molecular weight shifted to a slightly higher value. This result

indicated a possible post-translational modification of the GTP-binding protein in CNFl-treated cells (Fiorentini et al. 1994; Oswald et al. 1994). CNF1-induced electrophoretic shift of Rho is not due to a block in prenylation (post-translational modification of the C-terminal ends of small GTP-binding proteins) nor to phosphorylation of Rho (Oswald et al. 1994). Incubation of HEp-2 cells with CNF1 could also block the cytotoxic activities of two bacterial toxins known to act on the Rho–GTP-binding protein, namely exoenzyme C3 and *C. difficile* toxin B (Fiorentini et al. 1995). It has been demonstrated that CNF1 directly modifies Rho in vitro without the need for cellular co-factors (Flatau et al. 1997). Microsequencing of CNF1-modified Rho shows the existence of a single modification in the CNF1-treated GTPase compared with wild type, which consists of the change of glutamine 63 to glutamic acid (Flatau et al. 1997). Therefore, CNF1 exerts a specific deamidase activity. An identical activity for CNF1 on Rho has concomitantly been reported using mass spectrometry (Schmidt et al. 1997). Specific deamidation is a hitherto undescribed activity for a bacterial toxin. Indeed, toxin enzymatic activities described so far are ADP–ribosylation, depurination, metalloprotease, glucosyl transferase and cyclic adenosine monophosphate formation.

II. Consequences of CNF1 Activity on Rho GTP-Binding Proteins

The amino acid equivalent to Rho glutamine 63 in p21 Ras is glutamine 61. Rho glutamine 63 is known to be an important residue for the intrinsic and Rho GTPase-activating protein (GAP)-mediated GTPase of Rho (Rittinger et al. 1997). Upon electrophoresis, both CNF1-treated Rho and mutated Rho (RhoQ63E) exhibit a mobility shift identical to that of CNF1-treated Rho (Flatau et al. 1997; Schmidt et al. 1997). In CNF1-treated Rho and RhoQ63E, nucleotide-association affinity is slightly decreased (Flatau et al. 1997; Schmidt et al. 1997), whereas the RhoGAP activity is totally impaired (Flatau et al. 1997; Schmidt et al. 1997). RhoGAP has been shown to activate GTP hydrolysis of Rho by introducing an elongated domain that contains (at its tip) an arginine residue into the regulatory molecule (Rittinger et al. 1997). This structure, called an "arginine finger", allows the stabilization of Rho glutamine 63 during the catalytic GTPase transition state and thus increases the intrinsic GTPase activity of Rho. By modifying Q63 into E63 in Rho, CNF1 impairs the role of the arginine finger of RhoGAP. Thus, CNF1 allows Rho to be permanently bound to GTP, enhancing the activity of Rho on Rho effectors. In this context, it is worth noting that cholera toxin ADP–ribosylates the arginine 201 residue of the heterotrimeric GTP-binding protein $G\alpha s$, which plays the role of the arginine finger in this G-protein. This hinders $G\alpha s$ from hydrolyzing GTP via destabilization of the glutamine residue required for the hydrolysis step (Bourne et al. 1991). It is therefore fascinating to observe that cholera toxin and CNF1 achieve the same goal, which is to permanently activate a GTP-binding protein, by blocking GTP hydrolysis activities through

manipulation of two different residues involved in the same reaction. The catalytic activity of CNF1 on Rho together with the cellular downstream effects produced by this toxin are outlined in Fig. 4.

The specificity of CNF1 for Rho rather than Rac or Cdc42 must be analyzed at this point. SCHMIDT and coworkers (1997) reported that CNF1 is able to deamidate Cdc42 and Rho in vitro. However, in this study, only 30% of available Cdc42 was deamidated (SCHMIDT et al. 1997). Using a high concentration of CNF1, it is possible to deamidate Rho, Rac and Cdc42 in vitro, although deamidation of Rac appears to be only partial, even at very high doses of the toxin. Therefore, in our study, it seems that the substrates of CNF1 in vitro are, in order of decreasing affinity, Rho, Cdc42 and Rac. We do not know which are the best substrates of CNF1 in vivo. The level of Rho and Cdc42 activation by CNF1 in cultured cells may depend on the cell type. Epithelial cells, such as HEp-2, show actin membrane ruffling (lamellipodia) and formation of filopodia upon CNF1 treatment (Fig. 2E). These actin filaments co-localize with myosin type 2 and fimbrin/plastin. The membrane folding elicited in epithelial cells by CNF1 (Fig. 2B) is quite different from classical membrane ruffling described as lamellipodia (or leading edges) containing essentially polymerized actin filaments (MITCHISON and CRAMER 1996). Using video microscopy, we observed that membrane folding induced by CNF1 is highly dynamic and often appears to be localized at one point or two points of the membrane edge (DONELLI et al. 1994; Fig. 2F). Displacement of this point (or other points) either clockwise or counterclockwise around the cell is observed (DONELLI et al. 1994). Macropinocytosis (demonstrated by the presence of vacuoles starting from an active spot of membrane folding and increasing in diameter, together with their migration to the perinuclear cell area via a spiral trajectory) is observed in CNF1-treated HEp-2 cells when they are sparse in the culture (Fig. 3A). This is reminiscent of observations that activated Ras (BAR-SAGI and FERAMISCO 1986) or Rac (RIDLEY et al. 1992) can induce micropinocytosis in isolated (but not confluent) cells. Formation of filopodia in HEp-2 cells treated with CNF1 is also observed during the first hours of intoxication, but there is a regression of these structures after a longer incubation. This suggests that Cdc42 is indeed activated in HEp-2 cells at the beginning of CNF1 treatment. A hierarchy among Cdc42, Rac and Rho has been described in Swiss 3T3 cells: Cdc42 activates Rac, which in turn stimulates Rho (NOBES and HALL 1995). Thus, it is possible that the activity of CNF1 on Cdc42 (despite the fact that this protein appears to be a poorer substrate for CNF1 in vitro) might be dominant in HEp-2 cells at the onset of the intoxication and that Cdc42 might stimulate Rac, which in turn triggers lamellipodia formation. This may be due to a better immediate availability of Cdc42 compared with Rho in HEp-2 cells (and maybe in epithelial cells in general).

After a prolonged incubation of HEp-2 cells with CNF1, considerable spreading, with the abundant formation of thick stress fibers together with focal adhesion points, is observed. This spreading can be interpreted accord-

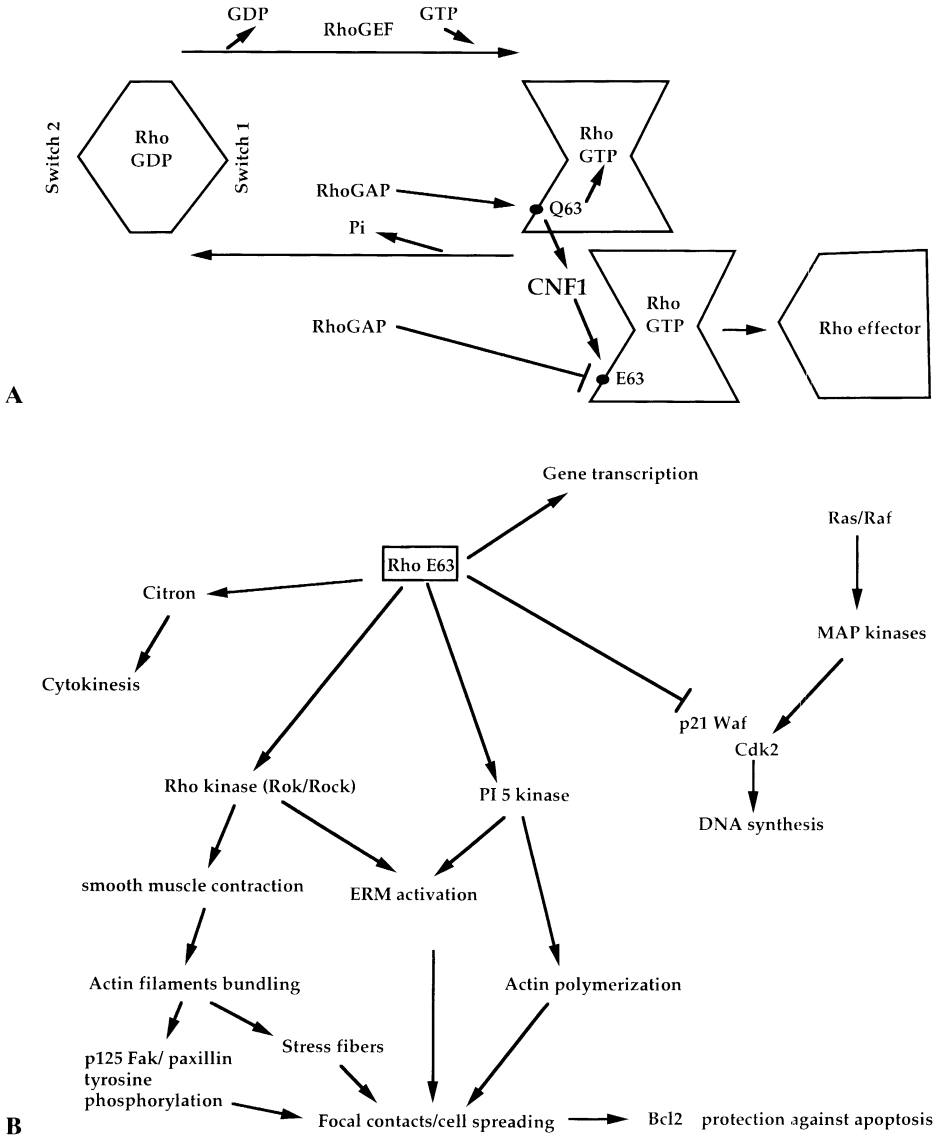

Fig. 4. Molecular mechanisms of cytotoxic necrotizing factor 1 (CNF1) on Rho guanosine triphosphate (GTP)-binding proteins, and downstream cellular effects of CNF1. **A** Rho, in its inactive state, is bound to guanosine diphosphate (GDP). Switch 1 and switch 2 are the two Rho domains that undergo structural modifications when the GTP-binding protein is bound to either GDP or GTP. Switch 1 is involved in the Rho downstream activation of an effector, whereas Switch 2 is required for the GTPase activity of Rho, with a pivotal role of glutamine 63. Rho guanine-nucleotide-exchange factor (Rho GEF) allows removal of GDP from Rho, which can bind GTP and becomes active with structural modifications of switches 1 and 2. Rho–GTPase-activating protein (RhoGAP) activates Rho glutamine 63, which induces GTP hydrolysis, causing the GTP-binding protein to go back to its inactive state. CNF1 deamidates Rho glutamine 63 into glutamic acid, with the consequence that RhoGAP is no longer active. Rho stays associated with GTP and permanently activates the downstream effector. **B** Schematic flow diagram of the possible cellular activities triggered by the CNF1-activated Rho E63

ing to the mechanism described as "actin-based motility" (Welch et al. 1997) and is due to activation of the Rho GTP-binding protein by CNF1. Indeed, cell motility or cell spreading on a support appears to be the consequence of the same molecular mechanism. According to the mechanism of actin-based motility, the cell leading edge is pushed forward by actin-filament polymerization at the plus end. Myosin motors, attached to the cell membrane and reading the actin filament to the plus end, pull filaments back, and this blocks the extension of the leading edge. If there is attachment of the actin filaments by focal contacts points, myosins can no longer pull backward-growing actin filaments and, thus, the leading edge is pushed forward. CNF1, by activating Rho, promotes the formation of focal contact points, probably by stimulating proteins of the ERM family (McKay et al 1997) known to link actin filaments with membrane receptors (Tsukita et al. 1997). CNF1 also stimulates the tyrosine phosphorylation of $p125^{fak}$ and paxillin (Lacerda et al. 1997), two proteins localized in focal contacts and playing important roles in signaling (Yamada and Geiger 1997). However, tyrosine phosphorylation of $p125^{fak}$ and paxillin is not a direct effect of CNF1-activated Rho, since it is dependent on actin-cytoskeleton integrity (Lacerda et al. 1997). Tyrosine phosphorylation of $p125^{fak}$ and paxillin is probably due to Rho-induced contractility implicated in the formation of focal contacts (Chrzanowska-Wodnicka and Burridge 1996). Furthermore, by increasing actin polymerization via PIP2 formation (Chong et al. 1994), CNF1 promotes cell spreading (Fiorentini et al. 1997a).

At this point, we must also state that transfection with the dominant form of Rho made by a mutation at position 14 (Rho Val14) gives rise to cells that do not undergo spreading. Indeed these cells are contracted and detach from the dish. This is also observed when the activated form of Rok is transfected in cells. Thus, the cell shape, resulting from either treatment with CNF1 or transfection with the activated form of Rho is not identical. Several factors can explain this result, but one must be emphasized. When cells are transfected with the activated form of Rho, they are overloaded with this regulatory protein and, thus, are clearly not in a normal physiological state. In these conditions, there may be a preferential activation of actin contractility induced by Rok, and a minor induction of focal contact points and PIP2 formation. CNF1, however, activates the normal cellular content of Rho proteins, allowing a more physiological response, i.e. a balanced activation of actin contractibility with focal contact formation and PIP2 production. Exposure to CNF1 induces a different phenotype in fibroblastic cells (such as mouse 3T3 cells or Vero cells) and epithelial cells. Usually, membrane folding, formation of filopodia or macropinocytosis is not observed upon treatment of 3T3 cells with CNF1. Rather, the formation of actin stress fibers and focal contacts resulting in cell spreading predominate (Fig. 2D). Thus, it seems that, in fibroblastic cells, Rho may be the immediate substrate for CNF1.

An interesting point remains to be discussed. Treatment of cells with CNF1 gives rise to multinucleation; however, we must emphasize that some cell species are more prone to multinucleate than others. Again, we observed

that epithelial cells (the best being HEp-2 cells, which reach 100% multinucleation after CNF1 treatment) are much more sensitive to the induction of multinucleation than are fibroblastic cells. Using video microscopy, we have analyzed how CNF1 induces multinucleation in HEp-2 cells (Donelli et al. 1994). Cells treated with CNF1 start a normal process of cell division, with chromosome condensation, nuclear-membrane fragmentation, rounding up and constitution of two daughter cells, together with an apparently normal cytokinesis process. This is observed by the constitution of a progressive cleavage furrow between daughter cells. Just before separation (formation of the midbody), the daughter cells are prevented from going further, and the release of the midbody gives rise to a binucleated cell. Our observation can be explained by the recent demonstration that a downstream effector of Rho named "citron", which contains a Rok-like kinase activity at its N-terminal end, co-localizes with Rho in the midbody (Madaule et al. 1998) and that the overexpression of citron gives rise to multinucleate cells (Madaule et al. 1998).

Although CNF1 impairs cytokinesis, it stimulates DNA synthesis (Lacerda et al. 1997). However, CNF1 does not activate the mitogen-activated protein (MAP)-kinase pathway (Lacerda et al. 1997). The mechanism of action of CNF1 on the activation of the cell cycle might thus be explained by the following cascade of signaling events: RasGTP, by activating Raf, activates the MAP-kinase pathway and triggers progression from the G1 to the S phase of the cell cycle. Excessive signaling from Ras/Raf induces the transcription of the Cdk2 inhibitor p21Waf1, thereby blocking the entry of cells into the S phase. When activated by CNF1, it has been shown that Rho blocks the inhibitory effect of Waf1/Cip1 on cell-cycle entry (Olson et al. 1998) and, thus, promotes cell division.

CNF1 does not induce protein kinase C (PKC) activation, inositol phosphate (InsP3) production or calcium mobilization (Lacerda et al. 1997). However, CNF1 activates the phosphatidylinositol-4-phosphate-5 kinase (PIP5K) associated with the cytoskeleton (Fiorentini et al. 1997a). The PIP5K induces the production of PIP2, a phosphorylated phosphatidyl inositide very important in the regulation of several actin-binding proteins, such as profilin and gelsolin. However, no accumulation of PIP2 could be observed in CNF1-treated cells (Fiorentini et al. 1997a). In keeping with these observations, it is known that this phosphorylated lipid is rapidly degraded in cells. Rho has been shown to activate the PIP5K in vitro (Chong et al. 1994). Thus, in addition to its effect on myosin contractility, CNF1 might also induce actin polymerization by stimulating the production of PIP2 and uncapping the barbed ends of actin filaments (Schafer et al. 1996).

Interestingly, the Rho subfamily of GTPases also controls gene transcription. Gene transcription and control of the actin-cytoskeleton organization are apparently accomplished by two different non-overlapping parts of the effector domains of small, Rho–GTP-binding proteins (Lamarche et al. 1996). Rac and Cdc42 control the c-Jun N-terminal kinase/P38 MAP kinase pathways

(MINDEN et al. 1995) by phosphorylating transcription factors (like extracellular signal-regulated kinases; BAGRODIA et al. 1995). However, Rho controls the binding of the transcription factor "serum-response factor" to the DNA-enhancer element "serum-response element" by an unknown, phophorylation-independent mechanism (HILL et al. 1995). As mentioned in the last section of this review, CNF1 is able to modify the pattern of expression of cell-adhesion molecules (FIORENTINI et al. 1998b).

E. Structure–Function Relationships of CNF1 and the Family of Dermonecrotic Toxins

I. The C-Terminal Part of CNF1 Contains its Enzymatic Activity

A hydrophobicity plot of CNF1 amino acids shows that this toxin contains two large, hydrophilic regions and two hydrophobic, sub-central domains partially overlapped by two predicted α-helices (LEMICHEZ et al. 1997). Comparisons of the predicted amino acid sequence of CNF1 with sequences of other toxins forming the so-called group of "dermonecrotic toxins" [CNF1, CNF2, dermonecrotic toxin from *Bordetella* (DNT) and the toxin from *Pasteurella multocida* (PMT)] has been analyzed (WALKER and WEISS 1994). They show that CNF1 and CNF2 share important regions of homologous amino acids with PMT in the N-terminal sequence, and a shorter region in the C-terminal sequence is homologous with DNT, produced by most members of the genus *Bordetella*. However, PMT and DNT are different and, as discussed later in this review, do not have the same catalytic activity. By using the GST gene-fusion method to produce recombinant N-terminal, 33-kDa and C-terminal, 31.5-kDa regions of CNF1, the functional organization of this toxin has been studied (LEMICHEZ et al. 1997). These experiments have clearly shown that the catalytic deamidase activity of CNF1 is located in the CNF1 C-terminal region, whereas the cell-binding properties of the toxin are on its N-terminal domain (LEMICHEZ et al. 1997; Fig. 5).

The homology of the amino acid sequences of CNF1, CNF2, PMT and DNT led us to assume that these toxins share the same general organization (LEMICHEZ et al. 1997). Dermonecrotic toxins are thus organized in a fashion similar to *Pseudomonas aeruginosa* exotoxin A (PAETA; ALLURED et al. 1986), although these toxins do not share the same catalytic activities, since PAETA is an ADP–ribosyltransferase toxin (IGLEWSKI and KABAT 1975). CNF1, CNF2, DNT and PMT bind to cell-membrane receptors. It seems that CNF1, CNF2 and PMT might bind to an analogous receptor, whereas the receptor for DNT would be different (LEMICHEZ et al. 1997). CNF1 and DNT share the same deamidase catalytic activity (FLATAU et al. 1997; HORIGUCHI et al. 1997; SCHMIDT et al. 1997), and CNF2 most likely also exhibits this activity. However, although PMT, like CNF1 and DNT, causes assembly of actin stress fibers and focal adhesion points, it does not induces a mobility shift of Rho. Discrepancies

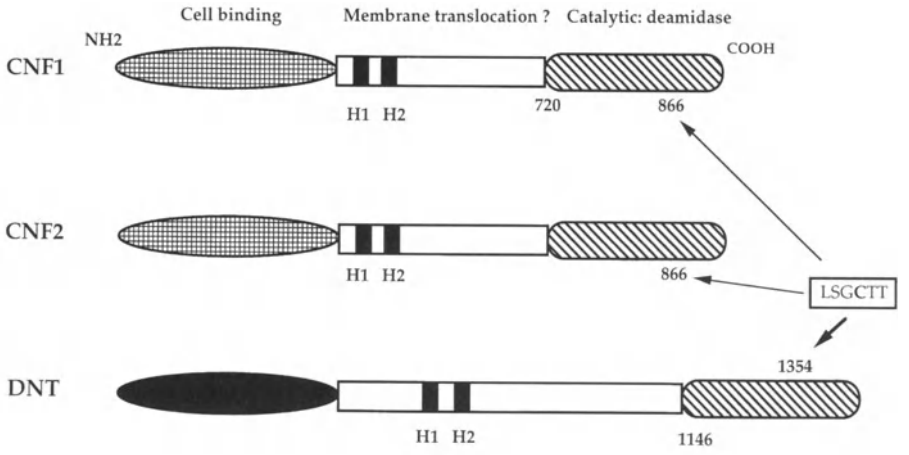

Fig. 5. Compared structure–function relationships of deamidating necrotizing toxins cytotoxic necrotizing factor (CNF)-1, CNF2 and dermonecrotic toxin (DNT). Cell binding, membrane translocation and catalytic domains of the three deamidating toxins. CNF1 and CNF2 probably recognize similar receptors on cells, whereas DNT binds to a different molecule at the level of the cell surface. The presence of two well-organized amphipathic helixes (H1 and H2) is found in the three toxins. A conserved motif encompassing the catalytic cysteine residue is localized on the C-terminal domain of the three toxins

among the amino acid sequences of the catalytic domains of CNF1, CNF2, DNT and PMT indicate that the *Pasteurella multocida* toxin has a different catalytic activity, which has yet to be characterized. In fact, in cells, PMT induces the formation of InsP3, mobilizes calcium and activates PKC (Lacerda et al. 1997). Thus, PMT most likely works upstream of Rho, probably activating a heterotrimeric G protein that controls the activity of phospholipase Cβ (Murphy and Rozengurt 1992). Curiously, it has been shown very recently that PMT might exhibit its intracellular activity on the large G-protein (Gαq)-coupled phospholipase, C-β1, via its N-terminal domain (Wilson et al. 1997, 1999).

C-terminal regions of CNF1,CNF2 and DNT are homologous, with a consensus sequence LSGCTT (amino acids 855–888 for CNF1 and CNF2, amino acids 1282–1315 for DNT; Lemichez et al. 1997), indicating that the catalytic center of these toxins might involve one or several of these amino acids. Recently, it was reported that, in vitro, CNF1 possesses transglutaminase, in addition to deamidase activity (Schmidt et al. 1998). This was shown by the ability of CNF1 to incorporate on RhoA Q63 alkylamines (Schmidt et al. 1998). However, the transglutaminase activity of CNF1 appears to be much lower than the deamidase activity (Schmidt et al. 1998). Furthermore, transglutamination of RhoA by CNF1 induced a downward shift of the GTP-binding protein (Schmidt et al. 1998), whereas deamidation of Rho A induces an upward shift (Fiorentini et al. 1994; Oswald et al. 1994; Flatau et al. 1997;

Schmidt et al. 1997), and microinjection of Rho E63 into cells reproduces all the characteristic features of CNF1. Because the active sites of transglutaminases involve a catalytic triad of cysteine, histidine and aspartic acid residues, these amino acids were recently analyzed in terms of CNF1 deamidase activity. As reported previously (Lemichez et al. 1997), the amino acid sequence 855–888 contains a cysteine residue (cysteine 866, belonging to the consensus sequence LSGCTT) essential for the catalytic deamidase activity (Schmidt et al. 1998). In addition, it has been shown that the conserved CNF1, CNF2 and DNT histidine residue at position 881 might also be involved in the catalytic activity of CNF1 (Schmidt et al. 1998). It has to be emphasized that it is impossible to reduce the catalytic domain (amino acids 720–1009) of CNF1 in a sizable manner without impairing the deamidase activity, suggesting that the conformation of the molecule is an important parameter for the catalytic activity (Lemichez et al. 1997).

II. The N-Terminal Part of CNF1 Contains its Cell-Binding Activity

The N-terminal domain of CNF1 (amino acids 1–299) contains the cell-binding region of CNF1 (Lemichez et al. 1997). This has been shown by the fact that a GST-fusion protein containing this CNF1 polypeptide competes with CNF1 on the cell receptor (Lemichez et al. 1997). Further work is now necessary to localize the cell-binding site of CNF1. Finally, the CNF1 domain, encompassing amino acids 299–720 and containing two hydrophobic domains (H1 and H2), is supposed to allow translocation of CNF1 across the cell membrane, as does the translocating domain of diphtheria toxin. Figure 5 summarizes, in a schematic representation, the structures of CNF1, CNF2 and DNT.

We actually know nothing about the nature of the cellular receptor of CNF1. It seems that CNF1, upon cell binding, enters the endocytic pathway, but the nature of this pathway is unknown. Whether CNF1 uses the coated pit/coated vesicle pathway like diphteria toxin (Moya et al. 1985) or, like the ricin toxin, is endocytosed by non-clathrin-coated vesicles (Moya et al. 1985), remains to be defined. It seems, however, that CNF1 must be transferred to an acidic compartment at some point of the endocytic pathway (late or early endosomes?) to exert its activity, since weak bases can block the toxic effects of CNF1 (Falzano et al. 1993; Lacerda et al. 1997).

F. Possible Roles for CNF1 as a Virulence Factor

I. CNF1 and Induction of Phagocytosis

How CNF1 interferes with host defenses is the question addressed in this paragraph. First, we have to recall that secretion of CNF1 by *E. coli* strains remains totally speculative. One hypothesis may be that CNF1 is not secreted but is simply released when the lysis of bacteria occurs, although *E. coli* is a "sturdy" bacterium, which (in contrast to *Streptococcus pneumoniae*, for instance) does

not lyse easily. We rather think there must be a system that allows secretion of CNF1 upon reception of a certain signal by the bacteria. The secretion machinery of CNF1 might belong to the type-3 secretion category (Hueck et al. 1997), since no signal sequence (type-2 secretion) or an adenosine triphosphate-binding cassette (type-1 secretion) associated with CNF1 can be found. Thus, at the present time, we must be prudent in describing CNF1 as a bona fide virulence factor for uropathogenic *E. coli*. However, incubation of CNF1 with different cell systems led us to suspect that this toxin could behave like an important bacterial virulence factor. Indeed, CNF1:

1. Induces an active macropinocytic activity in non-professional phagocytes, such as epithelial cells (Falzano et al. 1993)
2. Interferes with the transepithelial migration of polymorphonuclear lymphocytes (PMNs) across epithelia (Hofman et al. 1998)
3. Protects epithelial cells against apoptosis (Fiorentini et al. 1997b, 1998a, 1998b)
4. Modifies epithelium permeability in certain cells (Gerhard et al. 1998)
5. Changes the phagocytic activity of human macrophages (Capo et al. 1998)

We detail below these mechanisms.

Induction of phagocytic behavior in epithelial cells by CNF1 was the first described property evoking a possible role for this toxin as a virulence factor (Falzano et al. 1993). Bacterial invasion is a widespread process by which a microbe can find both shelter against the host defenses and an advantageous milieu for its growth. Furthermore, invasion of epithelial cells can lead to the passage of bacteria from the lumen of mucosa to the blood stream. For instance, *Salmonella typhi,* a Gram-negative enterobacterium, can invade epithelial cells, replicate within them and transcytose to the blood stream to disseminate to various organs (Galàn and Bliska 1996). Invasion of a cell by a microbial pathogen requires several steps governed by key virulence factors. This process requires binding of the bacteria to the cell surface first and then induction of macropinocytosis. In the case of *Salmonella*, it has recently been shown that, upon binding to cells, the bacterium introduces an exchange factor (SopE) for the small GTP-binding proteins Cdc42 and Rac (Hardt et al. 1998) into the cytosol through a type-3 mechanism, leading to activation of these GTPases. This results in the formation of membrane ruffles, which capture bacteria by provoking ruffles to engulf them, a process called "triggered phagocytosis" (Hardt et al. 1998). CNF1 uses the same strategy in *Salmonella* albeit by a different molecular mechanism. CNF1, by deamidating Rho (and perhaps Cdc42), activates it permanently and thereby triggers the formation of membrane actin folding that is able to capture latex particles (Falzano et al. 1993; Fig. 3B) or non-invasive bacteria (Falzano et al. 1993). Again, it is remarkable to observe that bacteria, by two divergent molecular mechanisms (activation by an exchange factor in *Salmonella* and point-activating mutation in *E. coli* CNF1) may achieve similar goals through stimulation of small GTP-binding proteins of the Rho family. It is interesting to point out that the CNF1

gene in uropathogenic *E. coli* is always found associated with the Hly-α gene (Blum et al. 1995). It is tempting to speculate that, if CNF1 triggers phagocytosis, Hly-α might allow the escape of the bacteria into the cytosol by rupturing the phagosomal membrane, as described for the pore-forming toxin listeriolysin during *Listeria monocytogenes* invasion (Cossart and Lecuit 1998). It has been reported that association of CNF1 and Hly-α is not more cytotoxic for human bladder cells in vitro (Island et al. 1998). However, in that study, whole bacteria were put in contact with cells and, thus, there was probably no release of CNF1 in the culture medium in these conditions.

II. CNF1 and Cell Apoptosis

Interestingly, the induction of phagocytosis by CNF1 can be correlated with the ability of the toxin to protect cells against apoptotic stimuli (such as ultraviolet radiation, UVB; Fiorentini et al. 1997b). The significant inverse correlation between the two phenomena suggests that they might be part of a pathogenic mechanism used by bacteria. In the mechanism used by CNF1 to hinder apoptosis, both proteins of the Bcl-2 family and mitochondrial homeostasis play a pivotal role. Indeed, we have shown that CNF1 is capable of reducing the mitochondrial-membrane depolarization induced by UVB and modulating the intracellular expression of some Bcl-2-related proteins. In particular, the amount of death antagonists, such as Bcl-2 and Bcl-X_L, increased following exposure to CNF1, while the amount of the death agonist Bax remained substantially unchanged (Fiorentini et al. 1998a). Bcl-2-family proteins and mitochondria play essential roles in apoptosis (Green 1998), and they are linked to one another. By modulating the expression of proteins of the Bcl-2 family (probably via Rho activation), CNF1 may operate on one of the main regulatory systems that drive cells towards death or survival. Very recently, we reported evidence that the Rho-dependent cell spreading activated by CNF1 is also involved in protection against apoptosis in epithelial cells (Fiorentini et al. 1998b). In addition to impairing nuclear fragmentation, CNF1 protects cells from radiation-induced rounding up and detachment and improves the ability of cells to adhere to one another and to the extracellular matrix by modulating the expression of proteins related to cell adhesion. In particular, the expression of integrins, such as α_5, α_6 and α_v, and of some heterotypic and homotypic adhesion-related proteins, such as the focal adhesion kinase, E-cadherin and α and β catenins, was significantly increased in cells exposed to CNF1. Thus, the toxin-induced improvement of cell adhesion and the promotion of Rho-dependent cell spreading are mechanisms clearly involved in hindering apoptosis in epithelial cells. This is in accordance with the recent opinion that cell spreading favors cell survival (Ruoslahti 1997). A toxin, such as cytochalasin B, which induces multinucleation and cell spreading without activating Rho and does not modify integrin expression is, in fact, ineffective in enhancing cell survival (Fiorentini et al. 1998b). Thus, by inducing both phagocytosis and protection against apoptosis, CNF1 might allow bac-

teria to invade epithelial cells and to prolong their survival to permit copious bacterial multiplication within them. Also, protection by CNF1 against apoptosis will allow cells to escape elimination by macrophages.

III. CNF1: Epithelial Cell Permeability and PMN Trans-Epithelial Migration

CNF1 has also been shown to reduce the transmigration of PMNs in intestinal T84 epithelial cell monolayers (Hofman et al. 1998). In the digestive tract, migration of PMNs across epithelia is a hallmark of disorders ranging from idiopathic, inflammatory bowel disease to bacterial enterocolitis (Yardley 1986). PMNs stimulated by bacterial chemoattractants transmigrate through epithelia via the paracellular space and enter the luminal space, where they can exert their scavenging activities. PMNs must force the occluding junction to reach the luminal space (Nash et al. 1987). In polarized epithelia, the Rho–GTP-binding protein regulates actin-filament organization at the apical pole of the cells and influences the permeability of tight junctions (Nusrat et al. 1995). Rho has been shown to regulate tight and adherin junctions in MDCK cells (Takaishi et al. 1997) and cadherin interactions in keratinocytes (Braga et al. 1997). When T84 cell confluent monolayers are incubated with 10 ng/ml of CNF1 for 18 h, no major changes in their trans-epithelial resistance (TER) is noted (Hofman et al. 1998). In addition, the absence of dissociation of the tight-junction-associated protein ZO-1 is seen (Hofman et al. 1998). However, CNF1 effaces the intestinal microvilli (as viewed by transmission electron microscopy), induces a strong reorganization of the actin cytoskeleton (especially at the baso-lateral face of cells) and reduces the transmigration of PMNs, as induced by the chemoattractant *N*-formyl-Met–Leu–Phe in either the baso-to-apical or apico-to basal-direction (Hofman et al. 1998). Thus, CNF1 might decrease PMN transmigration by remodeling the actin cytoskeleton without gross modification of tight-junction permeability. This mechanism would provide an advantage to the colonizing bacteria by reducing the ability of PMNs to cross the paracellular pathway. Recently, using Caco-2 cell monolayers, which exhibit a much lower TER on confluence (about 200 ohms/cm^2) than do T84 monolayers (1200 ohms/cm^2), it was shown that high concentrations of CNF1 (100 ng/ml) were able to partially decrease their TER (i.e., by ~40%; Gerhard et al. 1998). Results from our unpublished experiments are in agreement with this study. High concentrations of CNF1 (50–100 ng/ml) also lead to a TER decrease in T84 monolayers (Hofman and Boquet, unpublished observations). In our opinion, the observation of Gehrard and coworkers (1998) is due to an over-effect of CNF1. Indeed, with 10 ng/ml of CNF1, a profound reorganization of the actin cytoskeleton can be observed – in particular, a clear breakdown of microvilli and a dense meshwork formation of actin filaments on the baso-lateral pole of the T84 cells, without modification of the TER (Hofman et al. 1998).

Moreover, CNF1 has recently been shown to induce dramatic morphological changes and a profound reorganization of the actin cytoskeleton in human monocytic cells, impairing the CR3 phagocytosis but not the FcR-mediated ingestion (Capo et al. 1998). By limiting integrin-mediated uptake of microbes (CR3), and thus in a fashion independent of any specific immune response, phagocytosis of *E. coli* may be blocked by the microbes' CNF1.

G. Conclusions

CNF1 is the first toxin shown to act in the cytosol by deamidation of a glutamine residue belonging to a small GTP-binding protein. Even though the role of CNF1 as a virulence factor for uropathogenic *E. coli* is not yet established, this toxin is a new useful reagent for cell biologists working on the mode of action of Rho GTPases. Together with Rho inhibitors (exoenzyme C3 from *C. botulinum*, *C. difficile* toxins A and B and other related toxins) CNF1, which activates Rho, constitutes the first toxin that can modulate the same intracellular target both positively and negatively (Aktories 1997; Fiorentini et al. 1998c). Finally, we would like to stress that CNF1 induces, at the protein level, an activating "point mutation" of small GTP-binding proteins of the Rho subfamily, exactly as carcinogenic factors at the level of the Ras DNA do (Barbacid 1987). We suggest that CNF1 (or related toxins) could act as carcinogenic factors (as long as the toxin is produced), thus supporting the Erlich hypothesis that some infectious diseases could be origin of malignant tumors.

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CHAPTER 17

Shiga Toxins of *Shigella dysenteriae* and *Escherichia coli*

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A. Profile of the Shiga-Toxin Family

I. Nomenclature and History

The Shiga toxins constitute a family of functionally and structurally related toxins produced by *Shigella dysenteriae* type 1 (also called Shiga's bacillus) and a subset of diarrheagenic *Escherichia coli* called STEC (for Shiga toxin-producing *E. coli*). Shiga toxins of STEC were formerly called Shiga-like toxins and are alternatively named Vero toxins (CALDERWOOD et al. 1996; KARMALI et al. 1996). We favor the Shiga-toxin nomenclature system based largely on historical precedence. Indeed, the prototype for the Shiga-toxin family, Shiga toxin produced by *S. dysenteriae* type 1, was first described in 1903 by CONRADI (CONRADI 1903) and, independently, by NEISSER and SHIGA (NEISSER and SHIGA 1903). Conradi made the seminal observation that lysates of Shiga's bacillus caused hind-leg paralysis when injected into rabbits.

Studies of Shiga toxin (Stx) between the years 1903 and the early 1980s can be divided into three discovery phases. The first period of investigations primarily entailed distinguishing the protein Stx from the endotoxin or lipopolysaccharide (LPS) of *S. dysenteriae* type 1 (OLITSKY and KLIGLER 1920), purifying and characterizing the toxin (DUBOS and GEIGER 1946; VAN HEYNIGAN and GLADSTONE 1953a, 1953b), and discovering that iron represses toxin synthesis (DUBOS and GEIGER 1946; VAN HEYNIGAN and GLADSTONE 1953b). The second stage of Stx analyses resulted in the finding that Stx targets vascular endothelium (TESH and O'BRIEN 1991), an observation which served as an explanation for the apparent "neurotoxicity" of this toxin, and the realization that Stx is cytotoxic for a number of cultured epithelial cell lines (VICARI et al. 1960). The third phase of Stx experimentation during this 80-year period led both to the discovery, by Keusch and colleagues, that Stx causes fluid secretion and enteritis in ligated rabbit intestinal segments (KEUSCH et al. 1972) and to the purification of Stx to homogeneity (O'BRIEN et al. 1992). Further experiments with purified Stx culminated in our current concept of Shiga toxin as a tri-functional molecule, i.e. cytotoxic for selected cells, enterotoxic for rabbit ligated intestinal segments, and lethal for mice, rabbits, and other animals (O'BRIEN et al. 1980).

That Stx might be a member of a family of toxins was first indicated by the observation that certain strains of *E. coli* produced a cytotoxin that could be neutralized by anti-Stx serum (O'Brien et al. 1982; O'Brien and Laveck 1983). With the discovery that a newly described food-borne pathogen, *E. coli* O157:H7 (Riley et al. 1983), produced this "Shiga-like toxin" (Johnson et al. 1983; O'Brien et al. 1983) came the realization that this cytotoxin was, in fact, the same substance as the Vero toxin first described by Konolwalchuk in 1977 (Konolwalchuk et al. 1977; O'Brien et al. 1983). The finding that a "Shiga-like" (hereafter called Stx1 or Stx2 toxin from *E. coli*) or Vero toxin was produced by a novel, hemorrhagic colitis (HC)-inducing *E. coli* isolate stimulated a renewed interest in the Stx of *S. dysenteriae* type 1. More importantly, the understanding that Vero toxins and the Stxs of *E. coli* were the same proteins led to the establishment of these toxins as the common link in the development of hemolytic uremic syndrome (HUS), a sequela that occasionally occurs after infection with STEC or Shiga's bacillus (Karmali et al. 1983, 1985). The ultimate proof of the relationship among the Stx family members came with the cloning and sequencing of the genes of each of the family members and the understanding that all of the proteins share a common structure, mode of action, and a similar (if not identical) receptor (O'Brien et al. 1992; Melton-Celsa and O'Brien 1998).

II. The Stx Family

1. Traits that Make the Members Part of a Family

The members of the Stx family identified to date are listed in Table 1 and include: Stx of Shiga's bacillus; Stx1 of STEC, which differs by at most one amino acid from Stx (Strockbine et al. 1988; Takao 1988) and is immmunologically indistinguishable from it; and Stx2 and variants thereof (Stx2c, Stx2d, and Stx2e), produced by STEC. The Stx/Stx1serogroup is immunologically distinct from Stx2 and its variants, as determined by Vero cell cytotoxicity

Table 1. Stx-toxin family members

Toxin type	Location of operon	Environmental regulation	Relative toxicity for mice	Relative toxicity for Vero cells
Stx	Chromosomal	Iron repressed	+	+++
Stx1	Phage or chromosomal	Iron repressed	+	+++
Stx2	Phage	None identified	+++	+++
Stx2c	Chromosomal	None identified	+++	+
Stx2d[a]	Chromosomal	None identified	+++	+
Stx2e	Chromosomal	None identified	+	+++

[a] Not activated.

neutralization assays that use polyclonal or monoclonal sera raised against holotoxin (Strockbine et al. 1985 and 1986; Perera et al. 1988).

Each toxin family member shares four characteristics, as established experimentally or by inference from the published nucleotide or deduced amino acid sequences. First, members of the Stx family are encoded by an operon that is comprised of an A-subunit gene proximal to a B-subunit gene, and the amino acid sequences deduced from the nucleotides in that operon reveal identities of 55–99.9% (Calderwood et al. 1987; De Grandis et al. 1987; Jackson et al. 1987a, 1987b; Strockbine et al. 1988; Takao et al. 1988; Weinstein et al. 1988b; Ito et al. 1990; Schmitt et al. 1991). Second, the toxin members have a similar structure; each toxin is comprised of a single enzymatically active A-subunit polypeptide of ~32 kDa and a pentamer of B subunits (~7.7 kDa each) that is required for binding to the receptor on the target host cell. The A subunit can be nicked by trypsin or furin to form A_1 and A_2 fragments that remain held together by a disulfide bridge. The A_2 component appears to non-covalently hold the B pentamer to the A_1 fragment, as assessed by X-ray crystallographic studies of purified Shiga holotoxin (Fraser et al.1994). Third, Stx toxins have the same enzymatic function, i.e., the A_1 fragment cleaves a purine residue from 28-S ribosomal RNA (Endo et al. 1987; Saxena et al. 1989) which, in turn, leads to a cessation of protein synthesis in the target cell and the ultimate death of the cell by apoptosis (Keenen et al. 1986; Inward et al. 1995; Kiyokawa et al. 1998). Fourth, the eukaryotic receptor for Stx family members is globotriaosylceramide (Gb3) except for the pig edema disease toxin, Stx2e, which preferentially binds to the longer glycolipid globotetraosylceramide (Gb4) (Jacewicz et al. 1986; Lindberg et al. 1987; Degrandis et al. 1989; Samuel et al. 1990). Because of the specificity of the Stxs for Gb3, Lingwood's group (Lingwood et al. 1998) is exploring the possibility of using Stxs to treat tumors that express Gb3.

2. Characteristics that Distinguish Stx Family Members

Within the Shiga-toxin family, individual members differ in four primary features. These distinguishing traits are summarized in this paragraph and are discussed in detail below. First, some of the operons of the family members are located on the bacterial chromosome, and some are on transducing phages (Table 1; Scotland et al. 1983; Smith et al. 1983; O'Brien et al. 1984, 1989; Marques et al. 1987; Lindgren et al. 1993). Second, expression of Stx and Stx1 is repressed by high concentrations of iron, whereas the production of members of the Stx2 serogroup is not iron regulated (Dubos and Geiger 1946; Van Heynigan and Gladstone 1953; Calderwood and Mekalanos 1987; Weinstein et al. 1988a, 1988b). Third, antigenicity and affinity for receptor not only differ between the Stx/Stx1 and Stx2 serogroups but also within the Stx2-related toxins (Strockbine et al. 1985, 1986; Perera et al. 1988; Samuel et al. 1990; Schmitt et al. 1991; Kiarash et al. 1994). Fourth, toxicity of Stxs for Vero and other tissue-culture cells and for mice varies among family members

(Tesh et al. 1993; Lindgren et al. 1994; Louise and Obrig 1995; Melton-Celsa and O'Brien 1998).

III. Role of Stxs in *S. Dysenteriae* Type 1 and STEC Disease

1. Pathogenesis of Infection Caused by Organisms that Produce Stxs

The two groups of bacteria that produce Stxs, *S. dysenteriae* type 1 and STEC, have both similar and distinguishing disease attributes. Shiga's bacillus has a low infectious dose 50% (Levine et al. 1973). The organism colonizes the large bowel by penetration and invasion of M cells, followed by spread to adjacent mucosal epithelial cells (Acheson and Keusch 1994). Symptoms typically include diarrhea, dysentery (blood and mucus in stools), fever and tenesmus (Levine et al. 1973; Acheson and Keusch 1994). The clinical manifestations of shigellosis are similar among persons infected with different species and serotypes of *Shigella*, except that Shiga's bacillus tends to cause more severe disease and, in a small portion of cases, *S. dysenteriae* type 1-infected persons develop a sequela called HUS (Acheson and Keusch 1994). HUS is a triad of signs that includes hemolytic anemia, thrombocytopenia, and kidney dysfunction (Kaplan et al. 1990). Patients infected with *E. coli* O157:H7, the prototypic STEC, also present with diarrhea or bloody diarrhea (called HC) and, less frequently, fever and cramps (Griffin 1995). The absence of these symptoms may be because STEC, unlike Shigellae, do not invade epithelial cells in vitro or the bowel in experimental models (McKee and O'Brien 1995; McKee et al. 1995). Rather, *E. coli* O157:H7 and related *eae*-positive organisms adhere to epithelial cells by an intimin-dependent mechanism (McKee and O'Brien 1995). *Eae* is the gene that encodes the *E. coli* attach-and-efface locus, whose product is called intimin. Nevertheless, infection with *E. coli* O157:H7, like infection with *S. dysenteriae* type 1, can precede development of the HUS (Griffin 1995). For individuals infected with *E. Coli* O157:H7, the rate of development of HUS is 5–10% (Griffin 1995). However, the frequency of HUS after infection with other serotypes of STEC is not well documented.

2. Associations Between Stxs and Development of HC and/or the HUS

The association of bloody stool with *E. coli* O157:H7 infection is well established (Griffin 1995) and is believed to be a consequence of the action of Stxs on the microvasculature in the intestines. The connection between HUS development and production of Stxs by an infecting organism was confirmed epidemiologically (Karmali et al. 1985; Griffin 1995) after Karmali's 1983 proposal of that link (Karmali et al. 1983). However, the only direct evidence that Stxs play a role in human disease is the study by Levine et al. (1973), in which the investigators infected volunteers with either wild-type Shiga's bacillus (invasive, toxinogenic), a non-invasive, toxinogenic mutant, or an invasive. non-toxigenic mutant. The results of the investigation indicated that, though

the organisms had to be invasive to cause disease, toxinogenic, invasive strains caused more severe disease than invasive but Stx-negative mutants. It should be noted that the study in humans was done before the realization that infection with *S. dysenteriae* type 1 can result in the development of HUS.

3. Findings with Animal and Tissue Culture Models that Support a Primary Role for Stxs in Virulence of Shiga's Bacillus and STEC

The series of studies with animal and cell-culture models described below support the hypothesis that Stxs play a critical role in the pathogenesis of diseases caused by *S. dysenteriae* type 1 and STEC. First, monkeys fed wild-type *S. dysenteriae* type 1 develop more severe vascular colonic damage, as evidenced by increased blood in the stool and a greater white cell influx in the large bowel compared with monkeys who ingest an Stx-negative version of Shiga's bacillus (Fontaine et al. 1988). Second, rabbits infected intragastrically with RDEC-H-19A (a rabbit enteropathogenic *E. coli* strain transduced with the stx_1-encoding phage designated H-19A) have more severe enteritis than rabbits inoculated with the wild-type RDEC-1 strain (Sjogren et al. 1994). Moreover, RDEC-H-19A-infected rabbits, in contrast to animals fed the parental RDEC strain, acquire vascular lesions that resemble those seen in HC cases in humans. Third, mice pre-treated with streptomycin (to reduce facultative bowel flora) and then fed certain STEC strains that produce Stx2 or Stx2-variants develop toxin-mediated renal-tubular necrosis and die (Wadowlkoski et al. 1990; Lindgren et al. 1993). Fourth, greyhounds inoculated parenterally with Stx toxins develop a disease that mimics HUS, i.e., the animals present with renal failure accompanied histologically by vascular lesions in the glomeruli (Fenwick and Cowan 1998). Fifth, Stxs act as potent cytotoxins for human renal-glomerular endothelial cells in vitro (Louise and Obrig 1994, 1995).

B. Stx Genetics and Expression

I. Location, Organization, and Nucleotide and Deduced Amino Acid Sequences of Stx Family Member Operons

As previously mentioned, the genes encoding various members of the Stx family are either located on bacterial transducing phages or on the chromosome (Scotland et al. 1983; Smith et al. 1983; O'Brien et al. 1984, 1989; Marques et al. 1987; Lindgren et al. 1993), with remnants of stx_1- or stx_2-converting phages often found in the latter case (Strockbine et al. 1988; O'Brien et al. 1989; Lindgren et al. 1993). In fact, the same type of toxin gene may be encoded on the chromosome in an STEC strain of one O type and on a toxin-converting phage in another O type. For example, *E. coli* O157:H7 strain 933 carries stx_1 on the chromosome (O'Brien et al. 1989) but, in an *E. coli* O26:H11 strain designated H-19, stx_1 is phage encoded (Scotland et al.

1983; SMITH et al. 1983; O'BRIEN et al. 1984). Furthermore, a single strain may carry one type of Stx gene on its chromosome and another type on a transducing phage; e.g., *E. coli* O157:H7 strain 933 harbors both an stx_2-toxin-converting phage and a chromosomal copy of stx_1 (O'BRIEN et al. 1989). The evolution of the toxin-converting phages is the subject of considerable speculation (WHITTAM 1998).

The first toxin genes to be cloned and sequenced were those of stx_1 (CALDERWOOD et al. 1987; DE GRANDIS et al. 1987; JACKSON et al. 1987b). With analysis of the stx_1 nucleotide and deduced amino acid sequence came the realization that the genes for the A and B polypeptides fell within an operon and that the arrangement of that operon was similar to that of the *ctx* operon for cholera toxin (LOCKMAN and KAPER 1983; MEKALANOS et al. 1983). Specifically, the stx_{1A} gene lies proximal to the stx_{1B} gene; between the genes, a gap of 12 nucleotides encodes a ribosome-binding site (rbs). The strength of this rbs is hypothesized to be greater than that of the rbs that precedes stx_{1A}, such that more copies of the B polypeptide are made than of the A polypeptide, with a resulting ratio of one A subunit to five B subunits in the holotoxin.

Although evaluation of the stx_1 nucleotide and deduced amino acid sequence did not reveal significant homology with any other known prokaryotic toxin, deduced amino acid sequence homologies between the $Stx1A_1$ polypeptide and the A or enzymatic chain of the plant lectin ricin were detected (CALDERWOOD et al. 1987). At that time, the precise modes of action of ricin and Stx were not understood, but it was known that both toxins inhibited protein synthesis in eukaryotic cells by a mechanism that involved catalytic inactivation of 60-S ribosomes. The finding of significant homologies between these two toxins, together with similarities in the effects of these prokaryotic and eukaryotic toxins on target cells, led to the discovery that glutamic acid 167, an amino acid with a homologue at the same site in ricin, is in the active site of the Stx1A polypeptide (HOVDE et al. 1988).

When the genes of several of the Stx family members were ultimately cloned and sequenced, the operon architecture and the homologies with ricin were found to be common themes (JACKSON et al. 1987a; STROCKBINE et al. 1988; TAKAO 1988; WEINSTEIN et al. 1988b; SCHMITT et al. 1991). Another feature revealed by sequence analysis of some of the Stx family members was the presence of a binding site for the *fur*-gene product in the promoter region upstream of *stx* and stx_1. Fur acts as a co-repressor to prevent transcription of the stx_1 operon in the presence of iron (CALDERWOOD and MEKALANOS 1987, 1988). No *fur*-box homologies were identified in the promoter regions of stx_2 or stx_{2c} (WEINSTEIN et al. 1988b; SUNG et al. 1990). The presence of a *fur* box in the promoter region of the Stx and Stx1 operons directly correlates with the documented repression of toxin synthesis in the presence of iron (DUBOS and GEIGER 1946; VAN HEYNIGAN and GLADSTONE 1953a, 1953b; CALDERWOOD and MEKALANOS 1987; WEINSTEIN et al. 1988a). Similarly, the absence of a *fur* box in the promoter of stx_2 and other stx_2 serogroup members

is consistent with the failure of iron to regulate these genes (WEINSTEIN et al. 1988b).

II. Regulation of Toxin Production

Although iron is an established repressor of Stx/Stx1 synthesis in vitro, the role of iron repression of Stx/Stx1 production in vivo has only been cursorily addressed. Furthermore, regulation of Stx2 production is not well understood. In one study, a stx_2 regulatory locus was identified from a stx_2-converting phage (MUHLDORFER et al. 1996), but the mechanism whereby the product of this locus functions to up-regulate toxin production was not determined. In that same investigation, the authors also concluded that the DNA-binding protein H-NS and growth temperature affected stx_2 synthesis significantly or slightly, respectively. A small effect of temperature on *S. dysenteriae* type-1 synthesis of Stx, but not phage-encoded Stx1, has also been described (WEINSTEIN et al. 1988a).

III. Toxin Purification

Several different toxin-purification methods have been reported and are reviewed elsewhere (O'BRIEN et al. 1992). Our current strategy for the purification of Stx1 and Stx2 is based on a two-step procedure that includes a monospecific or monoclonal-antibody affinity column followed by passage of the eluent over a Sephadex G100 gel-filtration column. For isolation of Stx1 from a cell lysate, we typically use cytotoxin-neutralizing, monospecifc, rabbit anti-Stx1 sera as our adsorbent. We initially used the Stx/Stx1-neutralizing monoclonal antibody designated 13C4, which recognizes the B subunit of Stx1 (STROCKBINE et al. 1985), but we found that we had to re-generate the column frequently. For purification of Stx2, we used our monoclonal antibody, 11E10, which recognizes the A subunit of Stx2 (PERERA et al. 1988), partially neutralizes the toxin in vitro (PERERA et al. 1988), and fully neutralizes its activity in vivo (WADOLKOWSKI et al. 1990; LINDGREN et al. 1993). The yield from this protocol (~50% optimally) appears to largely depend on the concentration of monospecific antibodies [taken from an immunoglobulin G (IgG) fraction] or monoclonal antibodies (purified IgG fraction from ascites) used to generate the columns.

Two other practical aspects of toxin purification are worth noting for those engaged in this endeavor. First, variation exists in the efficiency of purification of the Stx2 serogroup members either by conventional or antitoxin purification approaches (O'BRIEN, DARNELL, and TWIDDY, personal observation). For example, we have not successfully isolated Stx2d with the anti-Stx2 monclonal column and have been forced to use a more standard approach for purification of this member of the Stx family. Second, attempts to generate monospecific, neutralizing anti-Stx2 by immunization of rabbits with chemically

toxoided Stx2 result in antisera that do not neutralize the cytotoxic action of the toxin (but have a high enzyme-linked immunosorbent assay titer) and do not serve as efficient adsorbents in an anti-toxin column. We speculate, based on sodium dodecyl sulfate polyacrylamide-gel electrophoresis analyses of our formalin-treated Stx2, that the B subunit of the molecule is destroyed by this procedure. The B subunit of Stx1 appears more refractory to dissolution by formalin and, perhaps as a consequence, it takes longer to chemically inactivate Stx1 than Stx2 (O'Brien and Twiddy, unpublished data).

C. Structure–Function Analyses of Stx Family Members

I. Structure of Stx

The determination of the crystal structures of the B pentamers of Stx1 (Stein et al. 1992) and Shiga holotoxin (Fig. 1; Fraser et al. 1994) revealed a number of characteristics of the toxins that had been predicted by other studies or

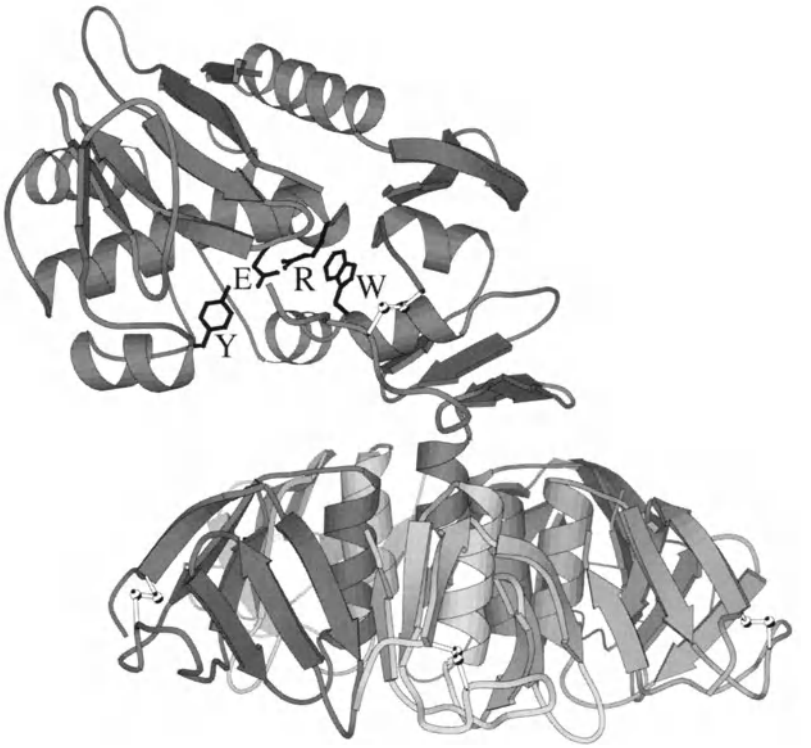

Fig. 1. A ribbon model of the crystal structure of Stx. The A subunit is shown above the B pentamer, with the side chains of four active site amino acids, Y114, E167, R170, and W203, indicated. This figure was drawn using the program MolScript (Kraulis 1991) and was generously provided by Marie Fraser, Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada

that were unanticipated. Traits of Stx/Stx1 that were evident upon X-ray-crystallographic analysis included:

1. The AB_5 subunit configuration
2. The similarity in structure of the Stx1 B pentamer with the B pentamer of heat-labile toxin (SIXMA et al. 1993)
3. The position of the A subunit compared with that of the B pentamer
4. The involvement of amino acid residues 77, 114, 167, 170, and 203 at the active sites of the toxins (Fig. 1; Table 2)
5. The importance of the A_2 peptide for holotoxin integrity
6. The apparent masking of the active site by a segment of A_2

Additionally, although FRASER et al. (1994) did not comment on the number of Gb3 molecules that could theoretically bind the holotoxin, STEIN et al. (1992) proposed that there was one binding site per B monomer, based on the crystal structure of the Stx1 B pentamer in the absence of Gb3. A later modeling study by Lingwood's laboratory suggested that there were two Gb3 binding sites per monomer (NYHOLM et al. 1996). Recently, however, the crystal structure of the Stx1 B pentamer coupled to a Gb3 analogue was published (LING et al. 1998) and, in that study, three non-cooperative Gb3-binding sites per B pentamer were described and named sites 1, 2, and 3. The integrity of sites 2 and 3 appears to depend on interactions between adjacent subunits. In contrast, site 1 is contained entirely within an individual monomer. Both the NYHOLM et al. (1996) and LING et al. (1998) studies agree on the approximate location of Gb3-binding sites 1 and 2. Site 3, however, was only identified by LING et al. (1998), who acknowledged that the affinity of site 3 is low. However, the tryptophan at position 34 of the B subunit, which is vital to site 3, had previously been shown to contribute to the toxicity of Stx (Table 3; JEMAL et al. 1995), a finding which suggests that site 3 may indeed have a physiological function.

II. Genetic Analyses of Stx Function

As noted previously, the homology between Stx and ricin, combined with knowledge of the crystal structure of ricin, led to identification of the Stx active site. The amino acids at residues 167, 168, 170, 202, 203, and 207 of Stx1 were found to be identical among several ribosome-inactivating proteins and in the active-site cleft of ricin (HOVDE et al. 1988). In that same study, HOVDE et al. showed that glutamic acid 167 was important for both the enzymatic and cytotoxic activities of Stx1. Subsequent mutations at position 167 in various Stx family members confirmed the importance of the glutamic acid residue (Table 2). Additionally, mutations at residues 170 and 203, as suggested by the homology to ricin, have been shown to be deleterious to toxin function (Table 2). The amino acids at positions 77 and 114, though not identified as homologous to residues in ricin, were found to contribute to full activity of the toxin (Table 2) and, subsequently were identified in the active site of the Stx crystal

Table 2. Mutations that affect the activity of the A subunit of the Stxs

Portion of A mutated	Specific mutation[a] (toxin type)	Effect of mutation	Reference
A_1	Y77S or Y77F (Stx1A)	Loss or 20-fold reduction in PSIA, respectively	Deresiewicz et al. 1992
	Y114F (Stx1A)	30-Fold reduced PSIA	Deresiewicz et al. 1993
	E167D (Stx1A) or E167D (Stx2)[b] or E167D (Stx2e) or E167Q (Stx1) or E167Q (Stx2e)	10^{2-3}-Fold reduction in PSIA; 10^{3-6}-fold-reduced VCC	Hovde et al. 1988; Jackson et al. 1990a; Yamasaki et al. 1991; Gordon et al. 1992
	E167L (Stx1)	10-Fold-reduced VCC; 40-fold-reduced PSIA	Yamasaki et al. 1991
	R170K or R170L (Stx1)	$2 \times 10^{2-3}$-Fold-reduced VCC; ~10^2-fold-reduced PSIA	Yamasaki et al. 1991
	R170K (Stx2e)	10-Fold-reduced VCC; fivefold-reduced PSIA	Gordon et al. 1992
	Y177S (Stx1A)	~10^3-Fold-reduced PSIA	Deresiewicz et al. 1993
	W203F or W203L or W203H (Stx1)	10–50-Fold-reduced VCC; 25–65-fold-reduced PSIA	Yamasaki et al. 1991
A_1–A_2 junction	C242S (Stx)	Altered cytotoxicity pattern; more sensitive to pronase; A_1 dissociated more readily from toxin-receptor complex	Garred et al. 1997
	R248A/R251G (Stx1A)	Delayed cytotoxicity	Burgess and Roberts 1993
	ΔS247–M252 (Stx)	Reduced cytotoxicity; still cleaved intracellularly to some extent	Garred et al. 1995
	R248H/R251H (Stx)	Delayed cytotoxicity; still cleaved intracellularly	Garred et al. 1995
	R247H or R250H (Stx2e)	No change in VCC or PSIA or mouse LD_{50}; still cleaved by proteases	Samuel and Gordon 1994
	R247H/R250H (Stx2e)	No change in VCC or PSIA or mouse LD_{50}; resistant to in vitro trypsin cleavage, but is cleaved intracellularly	Samuel and Gordon 1994
A_2	D278K (Stx)	10^4-Fold reduction in VCC; greatly reduced association with the B subunit	Jemal et al. 1995
	R288E (Stx)	10^5-Fold reduction in VCC; greatly reduced association with the B subunit	Jemal et al. 1995

PSIA, protein-synthesis-inhibition activity; *VCC*, Vero cell cytotoxicity.

[a] Each mutation is indicated by the single-letter amino acid designation for the wild-type residue followed by the position of that residue in the mature protein followed by the single-letter amino acid designation for the amino acid to which the residue was changed. The only exceptions are for deletion mutations, which are indicated by the Δ symbol.

[b] The original designation for this mutant was E166D but, because of a mistake in the original published sequence of Stx2, we chose to follow the numbering for the corrected sequence (Schmitt et al. 1991).

structure (FRASER et al. 1994). One residue, identified by mutation, Y177S (Table 2), does not appear in the active site of the toxin but may be involved in proper folding or orientation of the active site, since it is near other residues in the active site. Later deletion analysis of *stx* defined residues 75–268 as the minimal amount of Stx that retains enzymatic activity (HADDAD et al. 1993). However, amino acids in A_2 are required for assembly and stability of the holotoxin (Table 1; HADDAD and JACKSON 1993; AUSTIN et al. 1994; JEMAL et al. 1995).

As was true for the A subunit, both genetic and crystallographic analyses identified a number of amino acid residues involved in B-pentamer function (Table 3). Early genetic studies implicated three consecutive aspartic acid

Table 3. Mutations that affect the activity of the B Subunit of the Stxs

Specific mutation[a] (toxin type)	Effect of mutation	Reference
D16H/D17H (Stx)	No VCC; no binding to Gal–Gal–bovine serum albumin	JACKSON et al. 1990
N16D (Stx2d) or N16D (Stx2c)	Antigenicity and VCC increased to that of Stx2	LINDGREN et al. 1994
D18N (Stx)	Receptor specificity altered to that of Stx2e; Gb_4 bound in preference to Gb_3	TYRRELL et al. 1992
F30A (Stx1)	10^5-Fold-reduced VCC	CLARK et al. 1996
R32C (Stx2) or R33C (Stx); R33D (Stx)	Much reduced or no VCC; much reduced or no binding to Gb_3 or Vero cells	PERERA et al. 1991; JEMAL et al. 1995
W34G or W34F (Stx)	10^2-Fold-reduced VCC	JEMAL et al. 1995
A42T (Stx2); A43T (Stx)	No VCC for the Stx2 mutant; 10^4–10^6-fold lower VCC for the Stx mutant; much reduced or no binding to Gb_3 or Vero cells	PERERA et al. 1991
K53I (Stx)	10-Fold-reduced VCC	JACKSON et al. 1990
K53-biotinylated (Stx)	10^3-Fold-reduced VCC; loss of binding to Gb_3	KHINE and LINGWOOD 1994
G59D (Stx2) or G60D (Stx)	No VCC for the Stx2 mutant; 10^5–10^7-fold lower VCC for the Stx mutant; much reduced or no binding to Gb_3 or Vero cells for the Stx2 mutant; equivalent binding to Gb_3 and Vero cells for the Stx mutant	PERERA et al. 1991
Q64E/K66Q (Stx2e)	Changes receptor specificity from Gb_4 to Gb_3 only	TYRRELL et al. 1992

VCC, Vero cell cytotoxicity.
[a] Each mutation is indicated by the single-letter amino acid designation for the wild-type residue followed by the position of that residue in the mature protein followed by the single-letter amino acid designation for the amino acid to which the residue was changed.

residues at positions 16,17, and 18 in the N-terminus of Stx as important for Gb3 binding (Table 3; Jackson et al. 1990b; Tyrell et al. 1992). These same aspartic acid residues were found to be involved in Gb3 interactions in the crystallographic study of the Stx1 B pentamer bound to a Gb3 analogue (Ling et al. 1998). Interestingly, these adjacent aspartic acids are thought to be involved in different Gb3-binding sites such that D16 is in site 2, D17 is in site 1, and D18 is in site 3 (Ling et al. 1998). Other important B-subunit amino acid residues (Table 3) identified by both genetic and crystallographic means include F30 (Clark et al. 1996) in sites 1 and 2 (Ling et al. 1998), R33 (Perera et al. 1991; Jemal et al. 1995) in site 2 (Ling et al. 1998), and G60 (Perera et al. 1991) in site 1 (Ling et al. 1998). Although neither A43 nor K53 appear to be involved in hydrogen-bond formation or hydrophobic interaction with Gb3 (Ling et al. 1998), mutation of either of these residues results in reduced Gb3 binding and cytotoxicity (Table 3; Perera et al. 1991; Khine and Lingwood 1994). Thus, residues A43 and K53 may influence Gb3 binding without direct interaction. Finally, the Q64E/K66Q double mutant of Stx2e changes its receptor preference from Gb4 to Gb3 (Tyrell et al. 1992). A model of the Gb4-binding specificity of Stx2e, based on Ling et al.'s (1998) prediction of Gb3-binding sites for Stx1, places Q64 and K66 in site 1 (Cummings et al. 1998; in this reference, the residues are numbered Q65 and K67 in order to line up the Stx1 and Stx2e amino acid sequences).

D. Intracellular Trafficking of the Shiga Toxins

An outline of the current model (Sandvig and Van Deurs 1996; Sandvig et al. 1997) of the predominant pathway by which Stx intoxicates sensitive cells is as follows: (1) the B pentamer of holotoxin binds to Gb3; (2) the entire receptor–holotoxin complex is endocytosed; (3) the complex moves by retrograde transport from the endosomal compartment to the trans-Golgi network to the Golgi to the endoplasmic reticulum (ER); and (4) the A_1 subunit is released in the cytoplasm, where it targets the ribosome (Endo et al. 1987). The binding of the Shiga toxins to Vero cells is influenced by the fatty-acid-chain length in the ceramide moiety of Gb3, such that Stx1 preferentially uses C22:1Gb3 while Stx2c interacts more productively with C18:1Gb3 (Kiarash et al. 1994). Whether such differences in Vero cell binding translate to subtle alterations in the pathology induced by these two toxins remains to be determined. Following binding of the toxin to Gb3, the toxin–receptor complex is endocytosed. Although Sandvig's group has indicated that the endocytosis occurs through clathrin-coated pits (Sandvig et al. 1989, 1991), a recent study indicates that the Stx1 B subunit may also enter the cell in a clathrin-independent manner (Schapiro et al. 1998). The point at which the A subunit of the toxin is nicked is not clear. We hypothesize that the A subunit may be nicked by an intestinal protease prior to step 1 above, since we detect such cleavage activity in crude mouse or human intestinal-mucus preparations

(Melton-Celsa and O'Brien 1996). Stx is also sensitive to cleavage by furin (Garred et al. 1995), a protease found mainly in the trans-Golgi network but also on the cell surface and in endosomes (Molloy et al. 1994). The step at which the nicked A subunit is reduced to separate A_1 from A_2B5 is unknown but probably occurs after the toxin arrives in the ER, since both the holotoxin and the Stx B subunit have been visualized within the ER (Sandvig et al. 1994; Sandvig and Van Deurs 1996). Menikh et al. (1997) have proposed that the C-terminus of the Stx A_1 subunit acts as a signal sequence which, at pH 7, promotes translocation of A_1 across the membrane of the ER.

E. Virulence/Toxicity Differences among the Stxs

Investigations of the toxicity of the Stxs have revealed that Stx2 and its variants are more toxic than Stx1 when injected in pure form into mice or when STEC is fed orally to Str-treated mice (Table 1; Melton-Celsa and O'Brien 1998). Stx2 is also about 1000-fold more toxic in vitro than Stx1 for human renal microvascular endothelial cells (Louise and Obrig 1995). Epidemiological data also support a more critical role for Stx2 than Stx1 in the development of HUS (Scotland et al. 1987; Ostroff et al. 1989). However, both *S. dysenteriae* type 1 that make Stx and *E. coli* that produce only Stx1 have been associated with HUS cases. We speculate that more Stx1 than Stx2 is required to produce disease and that such levels of Stx1 are less frequently achieved either because: (1) not enough Stx1 is produced by the infecting organism or (2) because Stx1, which binds more avidly than Stx2 to Gb3 (Tesh et al. 1993; Obrig 1998), is not transported as efficiently as Stx2 intracellularly or systemically.

F. Immune Response to Stxs, Passive Anti-Stx Therapy, and Vaccine Development

I. Anti-Toxin Responses during STEC Infection

Data on the frequency of cytotoxin-neutralizing-antibody responses from individuals infected with Stx2-producing STEC are not available, because the presence of Stx2-interfering substances in human sera causes false-positive neutralization-assay results (Barrett et al. 1991; Bitzan et al. 1993; Rowe et al. 1993; Scotland et al. 1994). By contrast, the anti-Stx1 response is measurable, and a subset of children infected with Stx1-producing STEC who develop the HUS mount a cytotoxin-neutralizing, anti-Stx1 response. However, the percentage of anti-Stx1 seroconverters reported among children varies by country, from a low of 9% in Germany (Bitzan et al. 1993) to 18% in Italy (Caprioli et al. 1992) to 55–59% in Canada (Karmali et al. 1985; Rowe et al. 1993) to a high of 61% in Argentina (Novillo et al. 1988). These percentages are in contrast to the levels of anti-Stx1 in uninfected children, reported as 0%

in Germany (Bitzan et al. 1993) and 3% in Italy (Caprioli et al. 1992). One explanation for the failure to observe a cytotoxin-neutralizing anti-Stx1 response in a larger portion of children with HUS is that the amount of toxin necessary to consistently elicit such a response may be at or close to a lethal dose.

That low-dose exposure to Stx1 may be protective is suggested by two lines of circumstantial evidence. First, HUS occurs less frequently in adults than in children, and the percentage of adults with anti-Stx1 neutralizing antibodies in their sera is considerably higher (11–30%) than in children (0–3%; Barrett et al. 1991; Caprioli et al. 1992; Bitzan et al 1993; Siddons and Chapman 1993; Scotland et al 1994). Second, in one study (Wilson et al., 1996), healthy dairy-farm workers and their families had higher frequencies of anti-Stx1 neutralizing antibodies than their age-matched counterparts in the general population. These titers occurred in individuals who have no indications of clinical illness, even though some of them were found to harbor STEC. The authors of this dairy-farm-family study concluded that subclinical (or mild) infections with non-O157 STEC acquired from dairy cattle are immunizing for children at a young age.

II. Passive Therapy with Anti-Stx Antibodies

If, as the accumulated evidence strongly suggests, the production of Stxs by *S. dysenteriae* type 1 or STEC is required for development of the HUS as a sequela of these infections, then passive administration of anti-Stx antibodies should ameliorate disease (if not protect the patient), depending on the timing of antisera administration. This hypothesis, which is in keeping with the evidence on other toxin-mediated systemic diseases such as tetanus, botulism, and diphtheria, is the basis for current efforts to develop such prophylactic products. Two approaches to development of anti-Stx1 and anti-Stx2 antibodies for passive therapy are currently underway. First, Gerald Keusch and colleagues are attempting to prepare hyperimmune anti-Stx1 and anti-Stx2 sera by injecting volunteers with toxoids (Keusch et al. 1998) and harvesting and concentrating their immunoglobulins. Second, we and our collaborators have prepared cloned humanized anti-Stx1 and anti-Stx2 for human use (Edwards et al. 1998). Although neither type of product has yet entered phase-I safety tests, the humanized monoclonal antibodies have been shown to protect animals against toxin (Edwards et al. 1998), and similar results are anticipated for polyclonal anti-Stx1 and Stx2 hyperimmune globulin.

III. Vaccine Development

No vaccine is currently licensed to protect individuals from infection or disease caused by *S. dysenteriae* type 1 or STEC. There are valid arguments for and against the need for such a vaccine (Levine 1998; Tauxe 1998). If such an approach is ultimately deemed worthy of financial investment by a company,

a key component for such vaccines would be Stx1 and Stx2 toxoids. We propose that Stx toxoids should be generated by either rendering holotoxins enzymatically inactive by genetic mutation or by isolating non-toxic subunits of toxins. Our experience with the difficulties of fully chemically toxoiding Stx1 (see above section on toxin purification) and Stx2e (Gordon et al. 1992) make us wary of the potential for reversion of Stx toxoids into toxic molecules.

Delivery of the toxoid vaccines could be in the form of inoculated or inhaled toxoid–intimin conjugates, injection of an O157:H7 polysaccharide–toxoid conjugate (Butterton and Calderwood 1998; Konadu et al. 1998b), or ingestion of attenuated organisms that express one or more toxoids or subunits of toxin. Tests of an O157 LPS conjugate to *Pseudomonas* exotoxin A as a predecessor for a similar vaccine conjugated to Shiga toxoid have passed phase-I safety tests (Konadu et al. 1998a).

G. Summary

The Shiga-toxin family comprises a group of A:B5-subunit toxins that have the unique attribute of sharing enzymatic function with a plant toxin, ricin. Furthermore, the fact that most of these toxins bind avidly to Gb3 has opened the possibility that Gb3-expressing tumors may be targets for elimination by Stxs. This potential therapeutic use for Stxs is in contrast to the negative effect these toxins have on human health by their contribution to the pathogenesis of HC and HUS. Continued efforts towards the understanding of the biology of the Stxs and the organisms that produce them and the development of effective therapies and vaccines will be important in our attempts to eliminate these diseases.

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CHAPTER 18

Clostridial Neurotoxins

H. BIGALKE and L.F. SHOER*

A. Introduction

Tetanus and botulism are long-lasting, severe and often fatal diseases caused by the eight serologically distinct proteins tetanus toxin and botulinum toxins A, B, C_1, D, E, F and G. Although the main effect of all of these toxins is a dysfunction of striated muscles, the symptoms of tetanus and botulism are diametrically opposed. Tetanus is characterized by recurring spasticity of antagonistic skeletal muscles, and it is these dramatic symptoms that have attracted physicians for centuries. Hippocrates remarked in his Aphorisms (380 BC) "Spasm supervening on a wound is fatal... Such persons as are seized with tetanus either die within four days, or if they pass these they recover" (ADAMS 1849). In ancient Egypt, tetanus was also well known. It was named after one of the predominant symptoms, "lock jaw" (NUNN 1996). A very accurate description of the clinical signs was given by Aretaeus (1st century AD), who lamented, "An inhuman calamity! An unseemly sight! But neither can the physician furnish any assistance, as regards life, relief from pain or from deformity" (ADAMS 1856). Sir CHARLES BELL, a British neurologist, was the artist who produced the famous drawing (Fig. 1) of a soldier suffering from severe tetanus (BELL 1824). Before muscle relaxants were introduced and intensive care was available, such patients were doomed.

Botulism is a disease at least as fatal as tetanus. The symptoms of botulism resemble those of other neurological disorders, and only in relatively recent times have they been associated with the distinct condition of botulism. Interestingly, food poisoning, which in retrospect can be assumed to have been caused by contamination with *Escherichia coli*, staphylococcal and clostridial organisms, had been recognized for centuries as the source of a variety of severe disorders. Practical instructions for the production of a deadly material (presumably botulinum toxin) were published by the Indian poison expert, Schanaqua, 400 years ago: "Fill the gut of a sheep with the blood taken from the carotid artery of a black bull, tighten both ends, and hang it in the shadow of a mulberry tree. When dried grind the content firmly. A pinch of the powder mixed with food will kill the consumer within three days." The first noteworthy description of clinical botulism (Fig. 2), unsurpassed to this day, has been handed

* Deceased, August 24th 1999

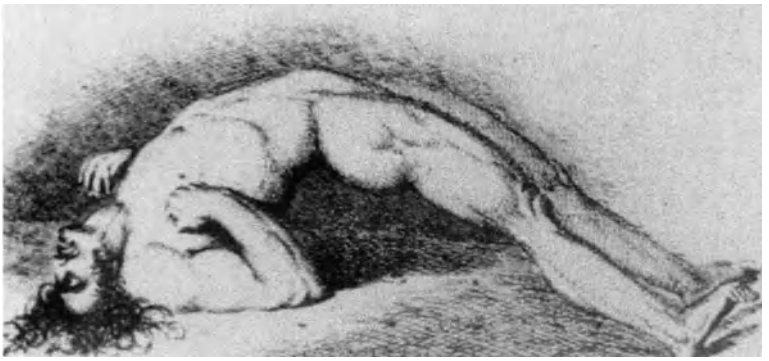

Fig. 1. Sir Charles Bell (1774–1842), the famous British physiologist and author of "New Idea of Anatomy of the Brain", which is known as the "Magna Carta of Neurology", painted this picture of a soldier wounded in the Peninsular War (1808–1814) and suffering from tetanus. Bell captured the characteristic symptoms in a brilliant manner. All skeletal muscles are in maximal contraction. The predominant neck and back muscles cause opisthotonus, and the predominant flexors lead to the bending of arms, fingers and toes. Characteristic symptoms are *trismus* (lock jaw) and *risus sardonicus* resulting from the contraction of the masticatory and facial muscles, respectively (from "Essay on the Anatomy and Philosophy of Expression", London, 1824)

down from the German romanticist and physician, Julius Kerner (Kerner 1817, 1820, 1822). He described not only the symptoms related to motoneuron failure but also those caused by dysfunctional vegetative nerves, such as the inability to produce earwax. He also experimented on animals to find the poison that "hindered the function of peripheral nerves that behave like a rusty wire conducting electricity" and even proposed its use for treating hyperkinesia.

Our knowledge of clostridial neurotoxins has mushroomed in recent years due to newly developed techniques and the impact the toxins have had as tools used in cell biology, molecular biology and pharmacology. Once the toxins' amino acid sequences became known (Eisel et al. 1986; Fairweather and Lyness 1986) speculations, soon to be proven correct (Sanders and Habermann 1992; Wright et al. 1992; Villiers et al. 1993), arose as to their possible enzymatic roles. Although synaptic proteins were biochemically well characterized, their function in exocytosis remained a mystery. It was not until the clostridial toxins were employed as tools that the role of synaptic proteins in vesicle turnover was revealed (Schiavo et al. 1992a, 1992b, 1992c, 1993a, 1993b, 1993c, 1994a, 1994b, 1995; Blasi et al. 1993a, 1993b; Hayashi et al. 1994; Yamasaki et al. 1994a, 1994b, 1994c). Eventually, the toxins lost their once nightmarish character to become drugs beneficial in the treatment of various neurological disorders (Scott 1989). Such an outcome had been anticipated by Kerner in 1822. They have even gained acceptance as lifestyle drugs, replacing surgery as a means to remove facial wrinkles (Carruthers and Carruthers 1992). Although many disciplines are involved in the investigation of clostridial toxins, this review focuses mainly on the pharmacological approach and the role the toxins play as tools in cell biology.


Das

Fettgift

oder

die Fettsäure

und

ihre Wirkungen auf den thierischen Organismus,

ein Beytrag

zur Untersuchung des in verdorbenen Würsten giftig wirkenden Stoffes,

von

Dr. Justinus Kerner,

Oberamtsarzt zu Weinsberg.

Stuttgart und Tübingen,
in der J. G. Cotta'schen Buchhandlung.
1822.

Fig. 2. Photograph of the 1822 paper by J. KERNER describing the symptoms of poisoning with sausages

B. Tetanus and Botulism in Man and Animals

I. Modes of Poisoning

Although seven serotypes of botulinum toxin are known, only types A, B and E play a major role in intoxication in man, while type F is of minor importance only. The other serotypes are involved in animal poisoning. For example, type C_1 predominantly affects birds (FRIEND and LOCK 1987). The routes by which the toxins gain access to the vascular system are probably the same in man and animals.

The toxins are produced by *Clostridium botulinum*, *C. barati* and *C. butyricum* under anaerobic conditions in protein-rich environments (VAN ERMENGEM 1897; BURKE 1919; HALL et al. 1985; MCCROSKEY et al. 1986; HATHEWAY 1989; MENG et al. 1997). When contaminated food is ingested, the pre-formed toxin is absorbed from the intestine. Although the toxins are proteins, they are probably protected against digestive enzymes by a complex consisting of hemagglutinins and nontoxic, nonhemagglutinating proteins

(Sugiyama 1980). In addition, the hemagglutinins appear to facilitate the penetration of the neurotoxins through the mucosal wall (Ohishi et al. 1977; Sugii et al. 1977; Fujinaga et al. 1997; Fu et al. 1998; Sharma and Singh 1998). However, botulinum neurotoxin (BoNT)/A complex can also be formed in the lower intestines of infants who have ingested food contaminated with the ubiquitous clostridial organisms. The latter colonize the intestine and cause infant botulism (Arnon 1980). A third mode of botulinum-toxin poisoning resembles that of poisoning by tetanus toxin. Like tetanus toxin, it can be synthesized in infected and poorly oxygenated wounds. Upon distribution within the vascular system, wound botulism (Burningham et al. 1994) will ensue.

II. Clinical Manifestations

The symptoms of botulism are common to all neuropathologic serotypes. Depending on the amount of toxin ingested, the first signs appear after 1–3 days. They are caused by inhibition of neuromuscular transmission at cholinergic synapses. Tightly controlled muscles, such as those in the eye, become paralyzed first. In severe cases, all striated muscles are enervated to some extent, and this leads to respiratory failure. Autonomic disturbances, such as mydriasis, dryness of the mouth and eyes, tachyarrhythmia, atonia of the intestine, and adiaphoresis, are further signs of the disease. In contrast to botulism, tetanus is characterized by rhythmic spasticities of striated muscles (Fig. 1). The forces attacking the tendons can be so strong that fractures of bones and the processes of vertebrae can occur. The therapy for both diseases is similar. Patients suffering from tetanus are first treated with a muscle relaxant. Irrespective of whether they are paralyzed by muscle relaxants or botulinum toxin, they then receive artificial respiration and intensive care, as required (Tacket and Rogawski 1989; Sun et al. 1994; Townes et al. 1996; Attygalle and Karalliedde 1997). The diseases can last from a few days in mild cases to several weeks in severe cases where all skeletal muscles are affected by spasticity and paralysis.

III. Pathophysiology

All symptoms caused by botulinum toxins are due to the inhibition of acetylcholine release from peripheral cholinergic, motor and autonomic nerve endings. Following absorption from the intestine, the toxins are distributed via the blood into the extracellular body fluid. It is not clear yet whether the neurotoxins alone, the whole complex or the individual complex components pass into the blood. In wound botulism, the entire complex is produced in the tissue. At physiological pH, however, the complex dissociates, and it is the free neurotoxin that causes the disease. Since botulinum toxin and immunoglobulins have similar molecular mass (MW) and charge, the volumes of distribution of both proteins are probably identical, i.e., 0.6 L/kg. It can be estimated from

therapeutic doses of BoNT/A that a blood concentration as low as 0.15 pg/ml causes recordable muscle weakness (Olney et al. 1988; Girlanda et al. 1992). Since concentration–response curves derived from human striated muscles in vivo are steep (Wohlfarth et al. 1997), one can expect that a 50- to 100-fold increase in concentration may be fatal. Thus, if applied parenterally, the lethal adult dose would be between 100 ng and 1000 ng of pure neurotoxin.

Based on in vitro experiments, a three-step model for the paralytic action of botulinum toxins was proposed (Simpson 1980, 1981). In the first step, they bind to unidentified peripheral, presynaptic receptors (Dolly et al. 1984; Black and Dolly 1986a, 1986b). (Binding sites have also been shown to be present in brain preparations (Habermann and Heller 1975; Kozaki 1979; Agui et al. 1983; Williams et al. 1983; Critchley et al. 1988; Kozaki et al. 1989; Herreros et al. 1997)). In the second step, the toxins are taken up into nerve terminals by receptor-mediated endocytosis (Black and Dolly 1986a, 1986b), which can be inhibited by chloroquin (Simpson 1982; Simpson et al. 1994). In the third step, muscles are paralyzed by the failure of nerve terminals to release the transmitter.

The main symptoms of tetanus are caused by the inhibition of glycine and γ-aminobutyric acid (GABA) release from inhibitory interneurons in the ventral horn of the spinal cord (Niemann 1991). It is likely that tetanus toxin is distributed into the same compartments that are entered by the BoNTs. Using data derived from an accident involving tetanus neurotoxin (TeNT)-contaminated blood (Rethy and Rethy 1997), the lethal human blood concentration of TeNT has been calculated to be in the same range as the lethal concentration of BoNT/A. Thus, the dose of TeNT lethal to an adult is also somewhere between 100 ng and 1000 ng.

Analogous to BoNTs, TeNT also binds to peripheral nerve terminals, where it is taken up by receptor-mediated endocytosis. From the nerve terminals, the toxin is actively transported within motor fibers to the ventral horn, where it finally arrives in interneurons. Thus, the whole toxin molecule passes through plasma membranes three times: (1) by entering and (2) by leaving motoneurons, and (3) by entering interneurons. Binding to interneurons, uptake into their cytosol and inhibition of transmitter release probably follow the three-step model discussed above for BoNT. Spasticity develops when release of inhibitory transmitter decreases. The lack of inhibitory input causes hyperactivity of α-motoneurons, and stimulation arriving from upstream is passed on, unfiltered, downstream. Optical, acoustical and tactile inputs generate the characteristic rhythmic convulsions.

The actions of clostridial neurotoxins have been studied in many in vitro models. It was found that all toxins of this group are capable of inhibiting the release not only of transmitters, but also of hormones; moreover, they obstruct the translocation of distinct plasma membrane proteins. Amongst the transmitters prevented from release by clostridial toxins are glutamate, GABA, glycine, noradrenaline, acetylcholine, various peptides and dopamine

(Ahnert-Hilger and Bigalke 1995). The toxins affect not only neuronal cells and preparations but also chromaffin cells (Bittner and Holz 1988; Marxen et al. 1989), pancreatic cells (Sadoul et al. 1997), parietal cells (Jöns et al. 1999) and macrophages (Pitzurra et al. 1989). While the toxic actions of TeNT and BoNTs at the neuromuscular junction resemble each other (Habermann et al. 1980; Dreyer 1989), the concentrations leading to the inhibition of acetylcholine release vary considerably. BoNT/A is 1000 times more potent than TeNT at this site. At central synapses, their potencies are reversed, and TeNT is 1000 times more potent than BoNT/A (Bigalke et al. 1981, 1985). Investigations regarding quantal release at the neuromuscular junction, however, have revealed that the mode of action by which the toxins inhibit transmitter release seems to vary also. Differences were obvious even amongst the BoNTs. While the transmitter release blocked by BoNT/A can be restored by increasing stimulation or applying black widow spider venom and various secretagogues, the blocks caused by TeNT and BoNT/B, C_1 and E are resistant to these manipulations (Dreyer 1989). Similar results were obtained in studies with chromaffin cells (Marxen et al. 1991a). Moreover, the nerve-evoked quantal release from TeNT- and BoNT/B, C_1, and E-treated nerve terminals is dissociated from stimulation, whereas, with type-A toxin, there is a correlation between stimulation and response (Dreyer et al. 1984, 1987; Bigalke, unpublished). It appears that the toxins interfere in different ways with steps leading to secretion. These steps have now been identified and will be discussed below.

C. Structure of Clostridial Neurotoxins

I. Genetic Determination

While TeNT is found in only one species, *C. tetani*, BoNTs are produced by at least six different species, including *C. butyricum* (Aureli et al. 1986; McCroskey et al. 1986) and *C. barati* (Hall et al. 1985; McCrosky et al. 1991). Moreover, there are strains that express more than one BoNT (Sugiyama et al. 1972; Hatheway et al. 1981; Gimenez 1984), while others carry the genes for two toxins but synthesize only one (Cordoba et al. 1995; Minton 1995). The *C. botulinum* strain C6813 contains a gene that encodes a hybrid toxin, H_N-L-chain/C_1-H_C/D (Ochanda et al. 1984; Morishi et al. 1996). Thus, unlike the TeNT gene which remains true to a single organism, the BoNTs' encoding genes are distinguished by their promiscuity. All strains, including *C. tetani*, may lose pathogenicity, which correlates with the loss of their toxin-encoding genes (Niemann 1991; Henderson et al. 1997). The genes encoding TeNT (Hard et al. 1977; Laird et al. 1980; Finn et al. 1984) and BoNT/G (Eklund et al. 1988) are associated with a plasmid. Those encoding BoNT/C_1 (Fig. 3) and D are found in bacteriophages (Inoue and Iida 1968, 1970; Vinet et al. 1968; Dolman and Chang 1972), and the genetic information for BoNT/A, B, E and F (Binz et al. 1990a, 1990b; Thompson et al. 1990; Kurazono et al. 1992) resides on the bacterial chromosome (Niemann et al. 1994).

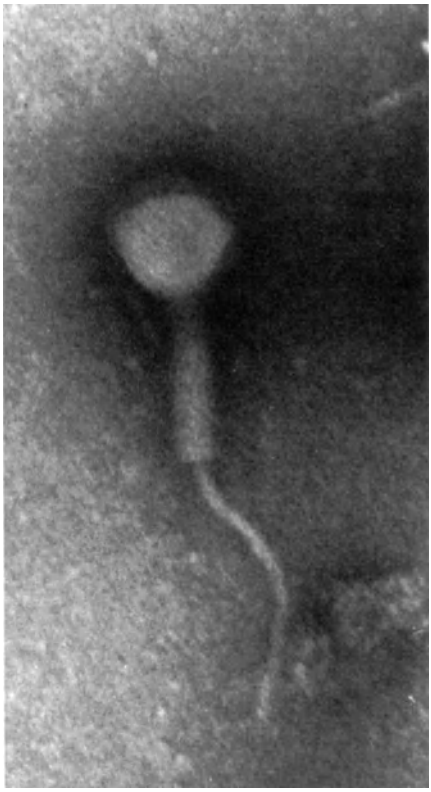

Fig. 3. Electron micrograph of a tox^+ bacteriophage encoding botulinum neurotoxin C_1 (courtesy of M.W. EKLUND, National Marine Fisheries Service, Seattle, Wash., USA)

II. Structure of Proteins

The clostridial neurotoxins belong to the so-called AB class of toxins. They consist of two separate parts with different functions. One part is the pharmacokinetic unit, which navigates the other part, the pharmacodynamic unit, through the plasma membrane into the compartment, where the latter performs its crucial action. Generally, the pharmacodynamic unit is an enzyme interacting with high specificity with only one or a few chemically related substrates. The pharmacokinetic unit, termed the heavy chain (H-chain), is the larger part of the molecule (MW = 100000 Da), while the enzymatic portion (MW = 50000) is referred to as the light chain (L-chain). The organisms produce the proteins as inactive single chains which, to become active, must be cleaved by limited proteolysis at a distinct "nicking site". Physiologically, nicking is performed by co-released clostridial proteases, which can, however, be replaced by trypsin or various other proteases (WELLER et al. 1988; HABERMANN et al. 1991). The resulting two proteins are connected by an interchain disulfide bond and ionic interactions and represent the actual neurotoxins (MW = 150000 Da). The neurotoxins can only be separated into their

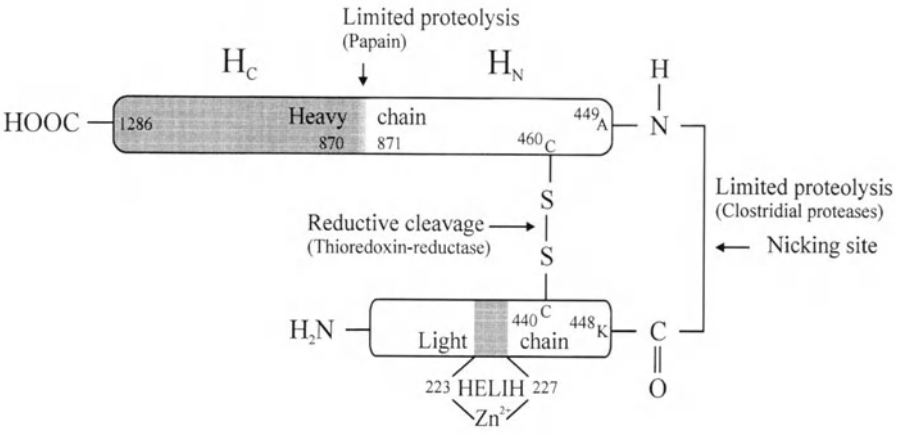

Fig. 4. Schematic structure of single-chain botulinum neurotoxin A. The numbers identify amino acid positions at which proteolytic cleavage and disulfide reduction occur (BINZ et al. 1990). The resulting di-chain molecule consists of the L-chain, in which the Zn^{2+} binding moiety is located, and the H-chain. The C-terminal portion (H_C) of the H-chain is responsible for binding. A fragment consisting of the N-terminal portion (H_N) of the H-chain attached to the L-chain (H_N-chain–L-chain) is formed when proteolysis at both susceptible sites occurs in the absence of reduction

chains if extreme conditions are employed, such as the application of a pH gradient. The reduction of the disulfide bond is not sufficient to accomplish this, because the chains are charged oppositely. The isoelectric points for L-chain–TeNT and H-chain–TeNT are 4.8 and 7.2, respectively (SATHYAMOORTHY and DASGUPTA 1985; WELLER et al. 1989). Once the chains are dissociated, toxicity vanishes, because the H-chain has no enzymatic activity, and the L-chain is not capable of penetrating membranes. Under appropriate conditions, the chains can be reconstituted to form active neurotoxin. Heterodimers of the H-chain and L-chain of different serotypes have also been prepared (WELLER et al. 1989, 1991). Figure 4 provides an overview of the structures of clostridial neurotoxins.

The botulinal neurotoxins, but not tetanus toxin, are embedded in a complex formed from hemagglutinins and a non-toxic, non-hemagglutinating protein. The complexes of different serotypes vary in molecular size (MW = 300 000–900 000 Da). They enhance oral toxicity by stabilizing the neurotoxins and, most likely, facilitate their absorption from the intestine, as recently shown for type A and C_1 neurotoxins (FUJINAGA et al. 1997; SHARMA and SINGH 1998). The composition of the complexes may contribute to the differences in oral toxicity observed in various animals (Gill 1982). Since tetanus toxin lacks stabilizing proteins, it is almost non-toxic when given orally.

Although serologically different, the clostridial neurotoxins share regions of highly conserved amino acid sequences. Toxins of the same serotypes produced by the same species are almost identical. However, heterology in the amino acid sequences increases in the same serotypes produced by different

groups of organisms. The overall amino acid homology among the different serotypes, including TeNT, is 30–40% (Eisel et al. 1986; Fairweather and Lyness 1986; Henderson et al. 1997). Both the L-chain and H-chain exhibit regions of highly conserved domains. All L-chains contain a common Zn^{2+}-binding sequence, HELxHxxH, which can also be found in various metalloproteases, such as thermolysin (Jongeneel et al. 1989; Schiavo et al. 1992a, 1992b, 1992c, 1993; Wright et al. 1992; De Paiva et al. 1993). The presence of Zn^{2+} is a prerequisite for the enzymatic activity of the L-chain (Sanders and Habermann 1992; Simpson et al. 1993; Adler et al. 1997). A membrane-spanning region, PYxGxAL, can be found in the H-chain of all neurotoxins (Henderson et al. 1997). Moreover, the H- and L-chains of all neurotoxins share the disulfide linkage that, in BoNT/A for example, connects Cys 440 (L-chain) and Cys 460 (H-chain). The C-terminal portions of the H-chains contain the greatest heterogeneity. They are involved in receptor binding, and their diversity may explain the lack of competition for binding sites between the different serotypes (Henderson et al. 1997).

All clostridial neurotoxins may have derived from a single ancestral gene. The diversity in amino acid composition that reflects mutations at the level of DNA may be a measure of the time elapsed since the toxin prototype emerged. With respect to the number of mutations, it is unlikely that Clostridia had originally produced the neurotoxins to kill mammals. It is more likely that an ancestral type of substrate (prototype substrate) also existed and occurred in "primitive" eukaryotes. Both the ancestral toxin and substrate have diverged to highly specialized components of prokaryotes and eukaryotes. One can conclude that, phylogenetically, clostridial neurotoxins (and probably all other protein toxins) followed the development of their respective substrates and that Clostridia continue to benefit from their ability to block exocytosis in mammals.

Recently, the crystal structure of BoNT/A was deduced (Lacy et al. 1998). The arrangement of the three-dimensional structure confirmed, for the most part, what had been predicted from the amino acid sequence and various experiments. The toxin consists of three spatially separated functional domains: the catalytic, binding, and membrane-spanning units, the last being responsible for translocation through membranes (Fig. 5). Uniquely, part of the translocation domain wraps around the enzyme like a belt. This may be the reason for the proteolytic inactivity of the non-reduced and non-nicked neurotoxin (Ahnert-Hilger et al. 1989b), because its active site is buried 20–24 Å deep within the protein and is accessible only through a channel shielded by the belt. The active site contains a Zn^{2+} which is coordinated by His 222, His 226 and Gln 261. One water molecule is bound by Gln 223. The entire unit is $55 \times 55 \times 62$ Å and is a mixture of α-helix and β-pleated-sheet secondary structures.

The binding domain contains two sub-domains: one binds to a ganglioside and the other to a protein receptor. They are spatially separated from the catalytic domain by the translocation domain; thus, their entire surface is avail-

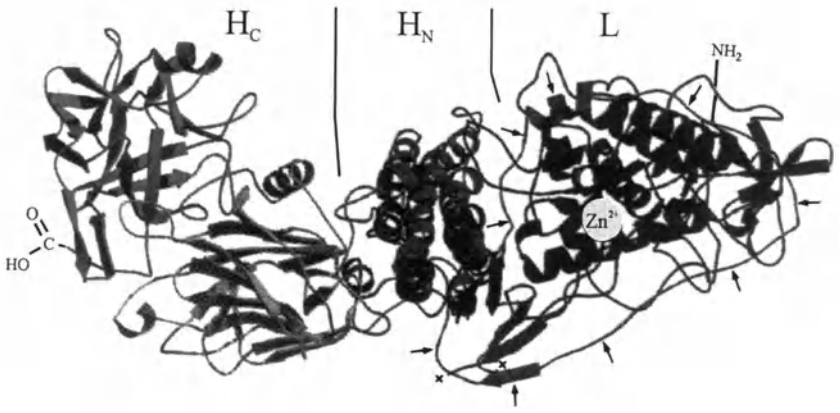

Fig. 5. Three-dimensional structure of botulinum neurotoxin A. The designations refer to the major components of the toxin, as described in Fig. 4. The Zn^{2+}-containing active site is buried within the catalytic domain. The nicking site between the L- and H-chains is indicated by "*X*". The translocation-domain belt wraps around the catalytic domain and is indicated by *arrows*. For a stereo view, see LACY et al. (1998) (courtesy of R. STEVENS, University of California, Berkeley, Calif., USA)

able for binding. Binding domains in BoNT/A and TeNT share structural homology (UMLAND et al. 1997). The differences in receptor recognition might be reflected by the different lengths of the C-terminal sub-domains. Both sub-domains share structural homology with lectins, serum amyloid P, β-glucanase and sialidase, all of which are known to bind to gangliosides that supposedly are also part of the clostridial neurotoxins' receptors (BIGALKE et al. 1986; MONTECUCCO 1986; MARXEN and BIGALKE 1989; MARXEN et al. 1989; NISHIKI et al. 1996). The similarity mentioned above between lectins and botulinum toxins may account for their competitive binding properties (BAKRY et al. 1991).

The clostridial neurotoxins must pass through endosomal membranes to gain access to the cytosol, where the substrate is localized. Translocation is accomplished at low pH by the membrane-spanning unit. Thus, a structural change in its conformation must take place when the pH decreases. The α-helices most likely to be involved in the translocation domain resemble those of coiled-coil viral proteins, human immunodeficiency virus 1 gp41/GCN4, influenza hemagglutinin and the MoMuLV TM fragment (BULLOUGH et al. 1994; Fass et al. 1996; WEISSENHORN et al. 1997; LACY et al. 1998), all of which penetrate membranes following pH-dependent structural changes. Although the proteins mentioned do not actually pass through pores, the conductivity of membranes may temporarily increase during penetration, thereby simulating pore formation. This has recently been demonstrated for TeNT (BIGALKE et al. 1996).

D. Toxicokinetics of Clostridial Neurotoxins

Using nerve–muscle preparations (Dreyer 1989; Simpson 1989), it was possible to divide the process of intoxication into three steps. In the experimental approach adopted, the muscle was used as an instrument for quantifying the actions of the toxins within the nerve endings. Although the conditions under which the toxins bind, pass through membranes and inhibit transmitter release were well characterized, the model did not provide detailed insights into the intracellular processing of the toxins. Accordingly, the finding that the final, paralytic step was heavily temperature dependent led to the somewhat simplified interpretation that it was an enzymatic reaction. It is now known for sure that neurotoxins are proteases. Although proteolysis can take place at temperatures as low as 20°C (Ahnert-Hilger and Weller 1993), paralysis in isolated nerve–muscle preparations (Schmitt et al. 1981) cannot occur. Therefore, the process of poisoning is probably more complicated than can be concluded from the experiments mentioned. In contrast with the in vitro situation, the interactions of enzymes and substrates in vivo must be preceded by additional events, such as an intracellular modification of the toxin. For example, whereas L-chain–TeNT is sufficient to cleave synaptobrevin-2 in test tubes (see below), it is necessary to employ the whole toxin to achieve an effect in intact cells.

I. Receptor Binding and Internalization

Clostridial neurotoxins enter the nervous system at the presynaptic region of the neuromuscular junction (Wernig et al. 1977; Dolly et al. 1984; Black and Dolly 1986a, 1986b) and at autonomic nerve endings (Ambache and Lippold 1949; Hensel et al. 1973). Apart from peripheral nerves, TeNT also binds nonspecifically to thyroid and renal tissues and pancreatic islet cells (Ledley et al. 1977; Eisenbarth et al. 1981; Habermann and Albus 1986). This binding, however, lacks any relevance for pathogenicity. In contrast to other tissues, neuronal tissue is rich in gangliosides that contain more than one neuraminic acid residue (Wiegandt 1985). TeNT and BoNT types A, B, C_1 and F bind preferentially to this group of gangliosides (Van Heyningen and Miller 1961; Simpson and Rapport 1971; Van Heyningen and Mellanby 1973; Ochanda et al. 1986; Kozaki et al. 1998). The ganglioside-recognition site is located in the C-terminal 34 amino acid sequence of the H-chain. This has recently been demonstrated for TeNT by using a ganglioside-photoaffinity ligand (Shapiro et al. 1997). TeNT and BoNT/A bind to different types of gangliosides (Marxen and Bigalke 1989; Marxen et al. 1991b). While the inhibitory action of TeNT is mediated mainly by type GD1b, BoNT acts via GD1a and GT1b (Ochanda et al. 1986; Marxen and Bigalke 1989). Consequently, these toxins do not compete with each other for the same binding sites (Williams et al. 1983; Dolly et al. 1984; Wadsworth et al. 1990). Clostridial toxins do not bind

the ubiquitous ganglioside GM1, which is the receptor for cholera toxin (Critchley et al. 1988; Niemann 1991). Binding of clostridial toxins occurs upon direct exposure to isolated nerve cells, brain preparations and nerve cell lines (Habermann and Heller 1975; Kitamura 1976; Dimpfel et al. 1977; Williams et al. 1983; Wellhoener and Neville 1987; Critchley et al. 1988; Li and Singh 1999). Moreover, clostridial neurotoxins bind to solid surfaces (Holmgren et al. 1980), lipid vesicles (Montecucco et al. 1989), artificial membranes (Yavin et al. 1987) and chromaffin cells (Marxen and Bigalke 1989; Marxen et al. 1989, 1991b) when these structures are artificially decorated with gangliosides. Even toxin-insensitive chromaffin cells become sensitized through this treatment, and hormone release is inhibited. Although clostridial toxins will bind to gangliosides in these systems, their binding affinities are low (Montecucco and Schiavo 1995) compared with those of nerve cells. For TeNT, at any rate, the binding constant is in the picomolar range (Wellhoener and Neville 1989).

Based on these observations, a double receptor was proposed in which one part is formed by the ganglioside, while the other part consists of a protein expressed selectively in neuronal tissue. Accordingly, nerve cell proteins were shown to be involved in toxin binding (Pierce et al. 1986; Parton et al. 1988; Schiavo et al. 1991). BoNT/B binds not only to gangliosides but also to the N-terminal portion of the vesicular protein synaptotagmin (Nishiki et al. 1994, 1996). The N-terminal part of synaptotagmin projects into the lumina of transmitter-containing vesicles (Nishiki et al. 1994). It is exposed to the synaptic cleft only during exocytosis, when vesicles fuse with the presynaptic plasma membrane. Since clostridial toxins bind not only to the releasing sites (active zones) but also to the entire membrane surface of nerve cells (Dimpfel et al. 1975, 1977; Bigalke et al. 1986), synaptotagmin may represent the high-affinity receptor involved in receptor-mediated endocytosis. The affinity of toxins for gangliosides is low. As gangliosides are present in such large numbers in nerve cell membranes, it is possible that they serve as acceptor sites for clostridial neurotoxins (Montecucco and Schiavo 1995). Binding can be almost abolished by neuraminidase, an enzyme that cleaves the neuraminic acid from gangliosides (Bigalke et al. 1986; Halpern and Neale 1995). If toxin-treated endplates are exposed to antitoxin antibodies, the bound toxin is neutralized, and muscle paralysis is ended (Schmitt et al. 1981; Dreyer 1989; Simpson 1989). From these findings, a two-step model of binding was developed (Montecucco 1986). In the first step, gangliosides filter the toxin from body fluids, where it is present in only minute amounts. Following exocytosis, the putative protein receptor responsible for internalization appears on the cell surface. In the second step, presynaptically enriched toxin binds with high affinity to this protein receptor.

Internalization into nerve endings takes place only when nerves are stimulated (Simpson 1980; Schmitt et al. 1981) and endocytosis occurs (Matteoli et al. 1996). Once taken up, the toxins cannot be neutralized by

specific antibodies (Schmitt et al. 1981). TeNT and BoNT/A occur in small vesicular structures (Parton et al. 1987), in cytoplasmic clathrin-coated (Black and Dolly 1986a, 1986b) and non-coated vesicles and in lysosomes (Schwab and Thoenen 1976; Montesano et al. 1982). It is likely that receptor-mediated endocytosis is involved in internalization, which is enhanced when nerves are stimulated and exocytosis occurs. As shown for BoNT/B, which binds to the intraluminal part of the vesicular protein synaptotagmin, the other clostridial neurotoxins may recognize different proteins that also protrude into the synaptic cleft after the fusion of vesicles with the plasma membrane. These proteins may be involved in the different routings of TeNT and BoNTs.

II. Translocation from Endosomes into the Cytosol and Priming

Toxins that are trapped within vesicles do not interfere with exocytosis, because their substrates are located in the cytosol. Translocation probably takes place when the vesicular contents are acidified. The inhibition of the vesicular proton-carrying adenosine triphosphatases (ATPases) by bafilomycin prevents the blockage of exocytosis (Simpson et al. 1994; Williams and Neale 1994; Matteoli et al. 1996). This observation supports the idea that the toxins probably undergo conformational changes when exposed to an acidic environment. Accordingly, insertion of both TeNT and BoNT into lipid bilayers occurs at low pH (Boquet et al. 1984; Roa and Boquet 1985; Menestrina et al. 1989; Schiavo et al. 1991). If the toxin is inserted into the membrane while in an environment with a lower pH than is present on the other side of the membrane, the toxin will be transferred into the higher-pH compartment (Bigalke et al. 1996). The entire toxin has been shown to pass through vesicular membranes resembling endosomal membranes. Penetration was accompanied by a change in the conductance of the membrane, simulating the opening of a pore. It is unlikely, however, that the L-chain can pass through this pore. Its unitary electrical conductance is between 28 pS and 35 pS. Thus, one can assume that the pores are too small for the large three-dimensional protein. Formation of ion channels of approximately the same size was also demonstrated to occur in planar bilayers (Hoch et al. 1985; Shone et al. 1987; Gambale and Montal 1988; Rauch et al. 1990; Schmid et al. 1993) and membranes of PC12 and nerve cells (Beise et al. 1994; Sheridan 1998), thereby giving rise to speculations about helical segments within the N-terminal portion of the H-chain (H_N). However, as recently inferred from its three-dimensional structure, the H_N of BoNT/A (Lacy et al. 1998) does not contain the predicted transmembrane, helical segment (Montal et al. 1992; Beise et al. 1994; Lebeda and Olson 1994). A region more likely to undergo conformational changes at different pHs is located distant from the residues suggested. The three-dimensional translocation domain was also found to be structurally distinct from that of other pore-forming toxins. It more closely resembles some coiled-coil viral proteins, that do not form pores but are able

to penetrate membranes and whose structures are pH dependent (LACY et al. 1998).

In the enzymatic reaction only the L-chain is involved (AHNERT-HILGER et al. 1989; BITTNER et al. 1989; STECHER et al. 1989a, 1989b; DAYANITHI et al. 1990; DEPAVIA and DOLLY 1990; LINK et al. 1992; SCHIAVO et al. 1992a, 1992b, 1992c, 1993; BLASI et al. 1993a,b). It is not clear, however, whether the entire toxin molecule or only the L-chain leaves the vesicular compartment and enters the cytosol. In any case, the disulfide bond connecting the H- and L-chains must be reduced to render the enzyme active. Whether the reduction leads only to conformational changes, unmasking the active site, or as a result of reduction, the chains are physically separated, remains to be elucidated. The H-chain must stay connected to the L-chain until it has gained access to the compartment where its substrate is located. Therefore, reduction must take place within this compartment. Since neither the H-chains nor the L-chains of clostridial neurotoxins have reductive capability, a neuronal enzyme must be responsible for the reduction of the disulfide bridge, which leads to the priming of the L-chain. A likely candidate for performing this enzymatic reaction is the thioredoxin–reductase system. This redox system, purified from brain (KISTNER and HABERMANN 1992) and chromaffin cells (ERDAL et al. 1995), cleaves the disulfide linkage between the chains. It is an endogenous neuronal enzyme that probably protects neurons from the destructive action of free radicals. With regard to the neurotoxins it converts the prodrug (the di-chain toxin) into the active drug (the L-chain).

Concerning the temperature-dependent process described above, which was observed in intoxicated endplates (SCHMITT et al. 1981; SIMPSON 1989), it turned out that at least three enzymes were involved (ATPase, reductase, protease). Since proteolysis by the L-chain occurs in vitro also at low temperature (AHNERT-HILGER and WELLER 1993), one of the other two enzymes present in the neurons may catalyze the temperature-sensitive step. This may be either the proton-carrying ATPase responsible for the acidification of endosomes or the thioredoxin–reductase. Blockage of the first enzyme protects exocytosis from inhibition. The direction of the reaction catalyzed by the second enzyme can be reversed by the addition of excess unreduced co-substrate, nicotinamide adenine dinucleotide cation. This leads to reassembly of the reduced H- and L-chains (KISTNER et al. 1993) into the inactive L-chain–S–S–H-chain (the di-chain form). Thus, if one of the enzymes fails at low temperature, paralysis will not occur.

Clostridial neurotoxins remain intact within cells over a long period of time. In nerve cell cultures, the half-life of TeNT was 3–5 days (HABIG et al. 1986), similar to the half-lives of L-chain–TeNT and L-chain–BoNT/A in chromaffin cells (ERDAL et al. 1995). Degradation is a slow process and occurs in autophagosomes. However, if only a few amino acids are removed, L-chain–TeNT loses its activity (KURAZONO et al. 1992; FAIRWEATHER et al. 1993). Exocytosis will recover when the toxins are degraded to inactive fragments. Clostridial neurotoxins may contain sequences that protect them from being

sorted into the lysosomal compartment, where proteolytic enzymes are located. A similar scenario has been demonstrated for immunoglobulin receptors (Mostov 1993). Following the intracellular inactivation of TeNT and BoNT/A in chromaffin cells with specific antibodies, exocytosis resumes within 3 days (Bartels and Bigalke 1992; Bartels et al. 1994). It has been concluded that the re-synthesis of substrates requires this length of time.

III. Sorting, Routing and Axonal Transport

Although clostridial neurotoxins have identical substrates, the clinical symptoms caused by TeNT and BoNTs are diametrically opposed. Their targets occur in all neuronal cells, but BoNTs and TeNT follow different anatomical routes. Once internalized by presynaptic nerve endings, TeNT finds its way into interneurons in the ventral horn of the spinal cord, while the BoNTs remain mainly in the peripheral nerve ending. However, in contrast to BoNTs, which do not provoke central actions when given peripherally, TeNT can also have peripheral effects, known as "cephalic tetanus". Although BoNTs do not affect central neurons, they also undergo retrograde axonal transport (Erdmann et al. 1975; Schwab and Thoenen 1976; Wiegand et al. 1976; Weller et al. 1986). Thus, despite many similarities, sorting and routing differ for the two toxins. Interestingly, two of the neurotoxins, TeNT and BoNT/B, cleave the same substrate at the same site (see below).

Clostridial neurotoxins probably use the physiological transport mechanisms employed by neurons for endogenous metabolic products (for example, for the transferrin receptor). Epithelial cells are equipped with similar transport mechanisms for the immunoglobulin receptors (Mostov 1993). Following receptor-mediated endocytosis, the receptor–immunoglobulin complex is sorted into trans-cytotic vesicles. These vesicles transport the complex to the apical membrane, where the immunoglobulins are released. In this case, it is the immunoglobulin receptor that carries the code for the selected route (Mostov 1993).

The preference for a distinct route is determined by the H_c fragment of neurotoxins. Only the holotoxin, H-chain and H_c fragment, and not the L-chain or H_N-fragment–L-chain, enter nerve endings, peripheral nerves and cell bodies of α-motoneurons or are taken up by neuronal-tissue preparations. Once internalized, the toxins, probably still bound to their respective receptors, enter the endosomal compartment, where sorting takes place. One portion of the BoNT molecules leaves the endosomal compartment at the nerve endings. This fraction of molecules produces botulism. Another portion of BoNT molecules and the TeNT molecules travel along a trans-cytotic route in transport vesicles from the apical region to the basolateral pole, i.e., from the nerve endings to the dendrites. For this purpose, the vesicles must be provided with a sorting signal. Since each clostridial neurotoxin binds to a distinct presynaptic receptor, the receptor itself may contain the code for route selection. This would explain the different behaviors of TeNT and the BoNTs at

the nerve endings and is in accordance with the trans-cytotic transport of immunoglobulin receptors in epithelial cells (Mostov 1993). Similar to immunoglobulins leaving epithelial cells, TeNT leaves the motoneurons (Schwab and Thoenen 1977; Schwab et al. 1979) and then enters interneurons, where it inhibits exocytosis (Osborne and Bradford 1973). To the BoNTs, however, the motoneuron is a dead end (Wiegand et al. 1976). They affect interneurons only if injected directly into the spinal cord (Hagenah et al. 1977). Since the adjacent nerve endings contacting the cell bodies of motoneurons are mainly those of inhibitory interneurons (Burke et al. 1971), TeNT blocks only inhibition and not excitation.

Release of TeNT from the soma of the motoneurons may occur by exocytosis, as shown for immunoglobulins (Mostov 1993). This type of exocytosis, however, is not inhibited by the toxin; otherwise, TeNT would not appear in the interneuronal space (Erdmann et al. 1981). The failure of BoNTs to leave the motoneuron may be explained by the higher sensitivity of motoneurons to this group of toxins. At any rate, at the level of the nerve endings, TeNT must be present at a concentration 1000 times as high as that of the BoNTs. The latter gain access to a substrate involved in secretion at the basolateral pole. BoNTs may inhibit exocytosis by cleaving this putative substrate in the soma of motoneurons, thus causing inhibition of their own release. The BoNTs themselves may be the agents preventing a TeNT-like action in the spinal cord. Figure 6 illustrates the route of the neurotoxins and their structural changes.

E. Toxicodynamics of Clostridial Neurotoxins

The L-chains of all clostridial neurotoxins are enzymes. Clostridial neurotoxin substrates have been found not only in motoneurons, but also in other cells of neuronal origin and in non-neuronal cells. Thus, the substrates, which have recently been characterized, may play a key role in cell function in general, while the toxins have become valuable tools in the study of intracellular trafficking.

I. Mode of Action of Clostridial Neuroproteases

All clostridial neurotoxins are metalloproteases that selectively cleave proteins involved in vesicle fusion (Montecucco and Schiavo 1995). The substrates occur at two locations: at membranes of small and large vesicles and in the presynaptic plasma membrane. They are termed soluble NSF-attachment protein receptors (SNAREs), where NSF stands for *N*-ethylmaleimide-sensitive fusion protein. NSF is a cofactor (an ATPase) necessary for fusion. It is currently believed that one v(vesicle)SNARE, synaptobrevin, is located mainly in the membranes of synaptic vesicles, and two t(target)SNAREs, syntaxin and SNAP-25 (synaptosome-associated protein of MW 25000 Da), associated with the plasma membrane, are involved in transmitter release. A small

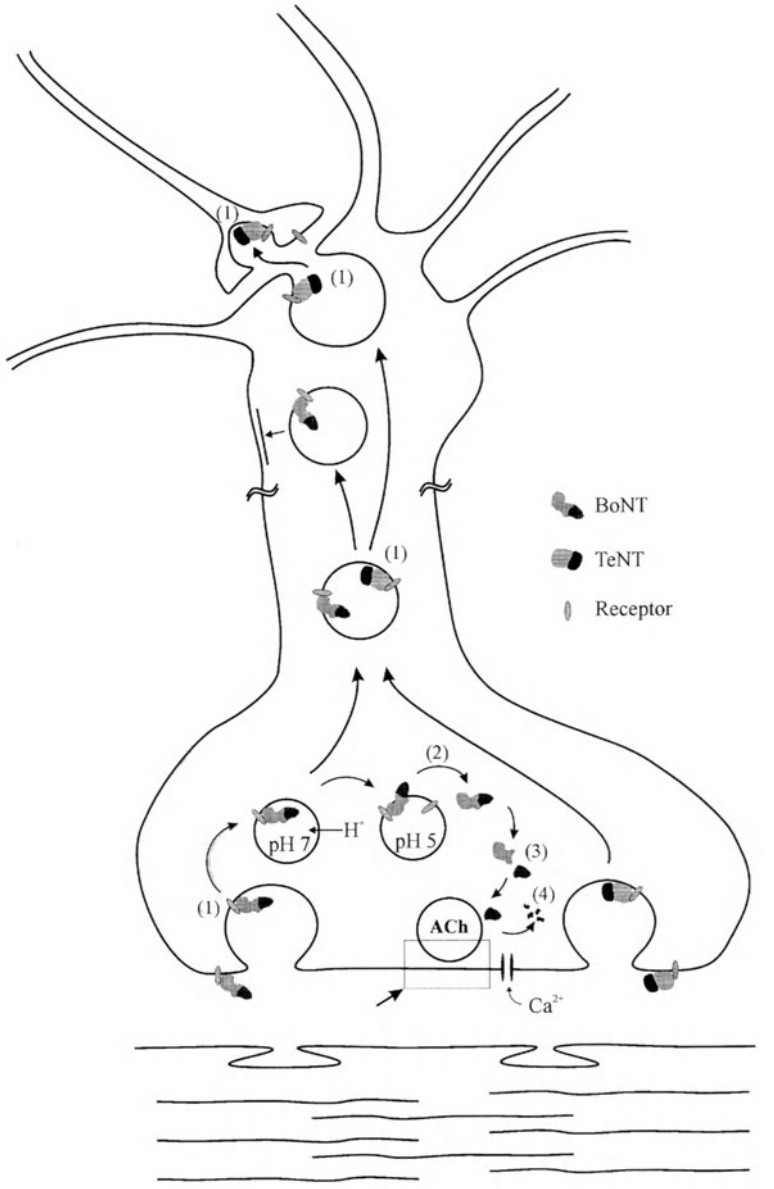

Fig. 6. Kinetics and metabolic fate of tetanus neurotoxin (TeNT). (*1*) Di-chain neurotoxins are taken up by receptor-mediated endocytosis and transported via axons into the spinal cord. TeNT is released from the soma and taken up by interneurons. (*2*) Following acidification of vesicles by an adenosine triphosphatase, botulinum neurotoxins (BoNTs) are released into the cytosol of motor-nerve endings, whereas TeNT gains access to the cytosol of interneuron presynaptic terminals. (*3*) Within the cytosol, both BoNTs and TeNT are primed by the thioredoxin reductase, and the toxins' L-chains cleave their respective substrates (Fig. 7). (*4*) Eventually, the L-chains are metabolized to inactive fragments, allowing the nerve terminals to recover

number of C-terminal residues of synaptobrevin and syntaxin are inserted into the membranes, while palmitoyl groups attach SNAP-25 loosely to cysteine residues in the plasma membrane. SNAP-25 does not possess a transmembrane region. The v- and tSNAREs form a complex that represents the core of the fusion machinery (SÜDHOF et al. 1993; HANSON et al. 1997; SUTTON et al. 1998). The proteins of the core complex are attacked exclusively by the L-chains of clostridial neurotoxins. They are capable of cleaving the substrates prior to complex formation. Once united, the SNAREs are not susceptible to the toxins (PELLEGRINI et al. 1994). If SNAP-25 is cleaved, a complex can still be formed, but this complex is not fully functional. If synaptobrevin or syntaxin are cleaved, even complex formation cannot occur. Since the formation of the complex is a prerequisite for fusion, the lack of complex formation leads to an inhibition of transmitter release. Moreover, release of insulin from islets of Langerhans and β-cell clones (SADOUL et al. 1995; LAND et al. 1997), growth-hormone secretion from pituitary cells (JACOBSSON et al. 1997), fusion of zymogen granules of the exocrine pancreatic cells (GAISANO et al. 1994) and translocation of the glucose transporter into fat cell membranes (CHEN et al. 1997) are also inhibited by TeNT and various types of BoNTs. This indicates a universal presence of either the "neuronal" SNAREs or toxin-sensitive SNARE isoforms. As yet, however, only one intracellular fusion event is known to be affected by a toxin (KIMURA et al. 1998). It is likely that intracellular fusion events involve different types of SNAREs (LINK et al. 1993) or toxin-insensitive isoforms (SADOUL et al. 1997).

Interestingly, secretion in yeast, although driven by similar SNAREs, is insensitive to the neurotoxins (FERRO-NOVICK and JAHN 1994; JAHN and SUEDHOF 1994). Assuming that, at one time, the prototype SNARE protein in the yeast's predecessor was susceptible to cleavage by the prototype toxin, the genes encoding the substrates and the toxins may have diverged from one another at a very early stage in evolution. From this point on, and due to a constantly changing environment, yeast and Clostridia no longer competed for nutrition, and the ability of clostridial toxins to prevent secretion in yeast was no longer advantageous for the survival of Clostridia.

L-chain–TeNT and L-chain–BoNT/B cleave synaptobrevin at an identical site (Gln 76). The L-chains of types D, F and G recognize the same substrate but split it at different locations (Lys 59, Gln 58 and Ala 81, respectively). L-chain–BoNT/A and L–chain BoNT/E cleave SNAP-25 at Gln 197 and Arg 180, respectively (MONTECUCCO and SCHIAVO 1995). L-chain–BoNT/C_1 is the only enzyme that acts on two substrates: syntaxin at Lys 253 and SNAP-25 at Arg 198 (VAIDYANATHAN et al. 1999). The high specificity of clostridial neurotoxins for their distinct substrates may derive from the fact that, for cleavage to occur, the three-dimensional structure of nearly the entire substrate is necessary. Truncated peptides or two-dimensional chains of the SNAREs cannot be cleaved (SHONE et al. 1993; NIEMANN et al. 1994; YAMASAKI et al. 1994a, 1994b). While the transmembrane region of synaptobrevin and the membrane anchor of SNAP-25 are not involved in enzyme–substrate interaction, the membrane-

spanning region of syntaxin, though hidden in the plasma membrane, is required for the enzyme to recognize it.

The cleavage of different substrates by the clostridial neurotoxins may explain why blockage by type A can be partially reversed, as shown for the neuromuscular junction (GANSEL et al. 1987) and chromaffin cells (MARXEN et al. 1991b; LAWRENCE et al. 1997). The blockage produced by the other toxins is always resistant to increased stimulation. Also, the differences in quantal release seen after poisoning with the different toxins may be due to the different targets. Thus, the complex may still function but does so less efficiently, even if a truncated SNAP-25 is incorporated.

II. Function of Substrates

SNAREs are involved in cell-vesicle trafficking (SÖLLNER et al. 1993; SCHELLER 1995; SÜDHOF 1995; CALAKOS and SCHELLER 1996). These pathways link the cell interior to the environment. They maintain the communication between cells, eliminate metabolic waste and transport cellular products from the location of manufacture to the point of consumption. Cell trafficking resembles the highly sophisticated logistics underlying the turnover of goods in a modern society. Both the single cell and modern society accomplish this task by using codes to define the target of the carrier and the fate of the goods. Thus, the intracellular transport systems (the vesicles) are decorated with signals that navigate them to a distinct destination (SÜDHOF 1995). Considerable information regarding vesicle trafficking has become available in recent years. Many studies have been conducted on the subjects of navigational codes, transport mechanisms, exocytosis and endocytosis (HANSON et al. 1997; JAHN and SÜDHOF 1999). This section discusses only clostridial neurotoxin-sensitive aspects of these subjects.

Transmitter-containing vesicles find their way to a distinct location at the presynaptic plasma membrane: the active zone, which is opposite to the folding zone of the postsynaptic site. This is where the acetylcholine receptors are located. At this site only, vesicles accumulate and fusion occurs, liberating the transmitter into the synaptic cleft. A similar route is chosen in central neurons. However, peptides, such as insulin, vasopressin and oxytocin, in addition to some monoamines, are also stored in vesicles and released upon stimulation. Whereas the latter class of substances is stored in large dense-core vesicles, low-molecular-weight transmitters are found in small synaptic vesicles. The two types of vesicles differ in their protein contents and their sites of manufacture. Dense-core vesicles are sealed off at the edge of the Golgi network, and small synaptic vesicles are derived from endosomes. Both, however, fuse with the plasma membrane through a common mechanism (JAHN and SÜDHOF 1994, 1999). Fusion of transmitter- or hormone-containing vesicles is regulated and requires Ca^{2+}, which is provided by voltage-activated Ca^{2+} channels (CHAPMAN et al. 1995; BANERJEE et al. 1996; MARTIN 1997). In nerve endings, they are in close proximity to the active zone. Another type of

vesicle translocates the glucose transporter in muscle and fat cells from the cytosol to the plasma membrane. This translocation, which is also regulated, does not require Ca^{2+}. These vesicles, however, share some features with small synaptic vesicles with respect to origin and protein composition (CAIN et al. 1992; HUDSON et al. 1993).

Following arrival at the active zone, vesicles must be primed for fusion. The proteins forming the core complex play an essential role in this event. Since the vSNARE is mainly located in vesicular membranes and the tSNAREs are found at the active zones, a model of fusion was proposed. In this model, the vSNAREs of the vesicles arriving at the plasma membrane form a tight ternary complex having a 1:1:1 stoichiometry with the tSNAREs, resulting in vesicle docking (HAYASHI et al. 1994). The three proteins bind to one another with high affinity and will neither dissociate spontaneously nor under denaturing conditions created by using the detergent sodium dodecyl sulfate (HANSON et al. 1997). The complex, however, is disassembled by NSF and soluble *N*-ethylmaleimide-sensitive-factor attachment proteins, called α-SNAPs (SÖLLNER et al. 1993; JAHN and SÜDHOF 1999). This resembles disassembly in yeast (BENNETT and SCHELLER 1993; ROSSI et al. 1997), where homologues of NSF are involved, again indicating the close relationship with respect to fusion among eukaryotes. The complex anchors the vesicles in close proximity to the site of fusion. Synaptotagmin, another vesicular protein closely connected to the complex, functions as a Ca^{2+} sensor (CHAPMAN et al. 1995). Upon depolarization and intracellular increase in the Ca^{2+} concentration, synaptotagmin was found to bind several calcium ions. Synaptotagmin then undergoes a conformational change and binds to phospholipids of the plasma membrane. Moreover, it interacts with one of the complex's proteins, syntaxin (BENNET et al. 1992). The primed vesicles fuse with the plasma membrane, thereby releasing their contents into the synaptic cleft. Thus, synaptotagmin works like a brake, preventing the primed vesicles from approaching the plasma membrane. The synaptotagmin brake on the vesicles can be released by Ca^{2+}. Following exocytosis, the membrane patches deriving from the vesicles are recycled by endocytosis within seconds (ROTHMAN and WIELAND 1996; KAISER and FERRO-NOVICK 1998; WARREN and MALHOTRA 1998; WENDLAND et al. 1998).

Another attractive model for vesicle fusion has recently been proposed (HANSON et al. 1997; SUTTON et al. 1998). It is based on the fact that both the t- and vSNAREs can be found in vesicular membranes (HANSON et al. 1997). Considering their high affinity for one another, it is unlikely that they would remain as single proteins in this membrane. It is more likely that a SNARE complex has already been formed; this complex, however, is nonfunctional. When Ca^{2+} is increased, α-SNAPs and NSF dissociate the vesicular complex. A new complex rapidly re-assembles from plasma membrane SNAREs and vesicle SNAREs. Vesicle membranes and plasma membranes are forced into close contact with each other by the contracting complex, thereby leading to fusion. Following this sequence of events, the complex itself initiates fusion

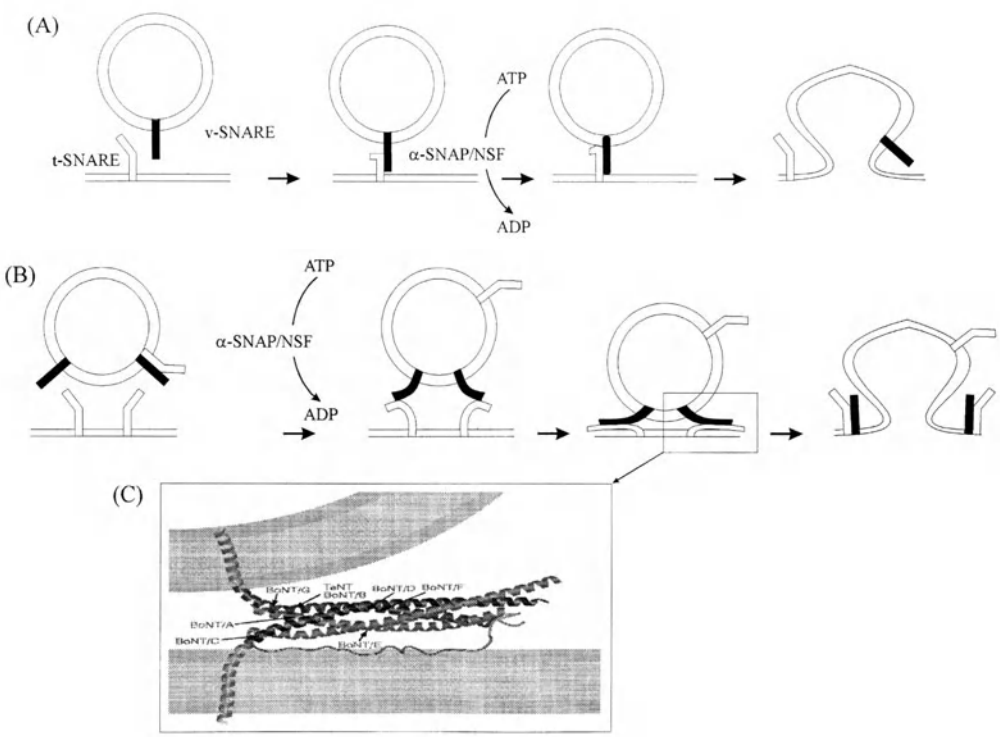

Fig. 7. Two suggested models regarding the roles which vesicle and target soluble *N*-ethylmaleimide-sensitive fusion protein (NSF)-attachment receptors (vSNAREs and tSNAREs) play in vesicle fusion. For simplicity, only a single vSNARE and tSNARE are shown. **A** In this model, the vesicle is docked to the plasma membrane by the complex of v- and tSNAREs. NSF disassembles and activates SNAREs. The activation causes vesicle fusion. **B** A non-functional SNARE complex is present in the vesicular membrane. Upon activation and disassembly of the vesicle-bound complex, a functional reassembly of the SNARE complex from two different membranes occurs. **C** A suggested structure of the fusion complex as it joins two membranes is presented. The locations of the neurotoxin-mediated cleavage sites are shown. The contracting coiled-coil complex forces the two membranes close together, initiating membrane fusion (courtesy of R. JAHN, Max Planck Institute, Goettingen, Germany)

(Fig. 7). Again, the patches are recycled and automatically harbor a nonfunctional complex. No matter which hypothesis will prove correct, fusion proteins are susceptible to clostridial neurotoxins, which are able to completely abolish transmitter release by cleavaging no more than a single type of SNARE.

F. Clostridial Neurotoxins Serve as Tools in Cell Biology and as Therapeutic Agents

For many years, TeNT has been used as a tool to gain insight into the mechanisms underlying hyperactivity in neuronal cell cultures (BERGEY et al. 1983,

1987). It can simulate epilepsy in an animal model designed to test potential anticonvulsants (FOCA et al. 1984; JEFFERYS and EMPSON 1990). Only recently, however, have the specific functions of the subunits (the L- and H-chains) become the center of attention. The L-chains have contributed to a better understanding of the distinct kinetic events leading to vesicle fusion in chromaffin cells (XU et al. 1998) and of the role played by fusion proteins in vesicle recycling. Using hippocampal cultures, the specific role played by Ca^{2+} in the toxin-sensitive steps of the exocytotic process has been identified (CAPOGNA et al. 1997). Further examples that illustrate the importance of toxins in this area of research include BoNT/E and F, which have been used to reveal the presence of SNAREs in pituitary cells and their involvement in growth-hormone secretion (JACOBSSON et al. 1997) and the role of SNAP-23 in the translocation of the glucose transporter (MACAULAY et al. 1997). Additional examples include TeNT and BoNT/A, both used to demonstrate SNARE-dependent exocytosis in neurohypophyseal cells (DAYANITHI et al. 1990, 1992). Through the use of neurotoxins, the processes of secretion in neuronal and a variety of non-neuronal cells have been shown to follow the same pattern. In these experiments, the holotoxins or their L-chains were artificially incorporated into the cytosol of renal tubular cells, adipocytes (CHEN et al. 1997) and various exocrine and endocrine cells (SADOUL et al. 1997), including pancreatic, enterochromaffin and adrenochromaffin cells (BITTNER and HOLZ 1988; MARXEN et al. 1989).

In all these examples, advantage was taken of the fact that the L-chains have a very high specificity for their respective substrates. The H-chains can also be utilized as tools (for example, in the labeling of specific neuronal connections; DIMPFEL et al. 1977). Their uptake and transport along specific transcytotic routes has been successfully employed in the retrograde axonal delivery of gelanin (JOHNSTONE et al. 1990) and glucose oxidase (BEAUDE et al. 1990). Thus, the H-chains can be used as carriers to transport active compounds to their target cells. The holotoxins are sorted into transport vesicles more efficiently than the isolated H-chains. This is, perhaps, due to the fact that structural changes in the chains result during chain separation (ZHOU et al. 1995). To circumvent this problem and deliver drugs (antivirals and growth hormones) into the spinal cord, recombinant inactive mutants of the holotoxin, expressed in *E. coli*, were used (ZHOU et al. 1995). Moreover, the hemagglutinins, which protect the neurotoxin from digestive enzymes during enteral absorption (CHEN et al. 1998; FU et al. 1998), may serve as possible vehicles for the transfer of protease-sensitive peptides from the intestine into the blood. Thus, neurotoxins have become valuable research tools in the study of secretory processes in general and neuronal exocytosis in particular. Table 1 provides a listing of the various types of commercially available preparations of neurotoxins, chains and derivatives.

Clostridial neurotoxins are extremely toxic to mammals by virtue of the fact that they cleave proteins that are crucial to neurotransmission. However, they are not cytotoxic. BoNT/C_1, which cleaves two fusion proteins (SNAP-25

Table 1. Distributors of clostridial neurotoxins

Company	Location	Web Site	Products
Alomone	Jerusalem, Israel	www.alomone.com	TeNT
Calbiochem	La Jolla, California, USA	www.calbiochem.com	BoNT complexes, chains, TeNT, fragments, derivatives, toxoids
List Biological Laboratories, Inc.	Campbell, California, USA	www.listlabs.com	Pure BoNTs, chains, TeNT, fragments, derivatives, toxoids
Sigma Chemical Co.	St. Louis, Missouri, USA	www.sigma-aldrich.com	BoNT complexes
Wako BioProducts	Osaka, Japan	www.wako-chem.co.jp	BoNT complexes

BoNT, botulinum neurotoxin; *TeNT*, tetanus neurotoxin.

and syntaxin), may be an exception, because it is capable of destroying neurons in culture (Williamson et al. 1996). Nonetheless, transmitter release does not seem to be important to single cells. It is only crucial to the whole organism. If the organism survives, the toxin is metabolized (Erdal et al. 1995), and exocytosis will recover. Lack of cytotoxicity and long-lasting action are prerequisites for the use of BoNTs as therapeutic agents (Montecucco et al. 1996). When locally injected into a dystonic or spastic muscle, BoNTs cause paralysis of the affected muscle. Therefore, the BoNT/A–hemagglutinin complex has been used for the treatment of neurological disorders, which are characterized by an unremitting overactivity of motoneurons and spasms of striated muscles, such as blepharospasm, torticollis spasmodicus, laryngeal spasms of the jaw muscles and many other forms of dystonia and spinal spasticity (Fig. 8). The toxin has been used to curb hyperactivity of salivary and sweat glands (Naumann et al. 1997), as many functions of autonomic nerves are mediated by acetylcholine. Moreover, the tone of smooth muscles can be decreased in patients suffering from achalasia and anal fissures, thereby reducing pain. Recently the toxin has even been employed to smooth facial wrinkles which are caused by contractions of platysma and facial muscles. In all these cases, only a few nanograms were injected into the muscle near the endplate region or close to the hyperactive glands. It is unlikely that an amount of protein that is sufficient to paralyze muscles at a distance from the injection site will diffuse out of the muscular depot. Thus, the toxin's effect is strictly limited to nerve endings in close proximity to the injection site. Systemic effects, though rare, are possible, particularly if a very high dose is applied or if the injection is off target.

Within 3–5 days, cholinergic transmission gradually diminishes, and the muscle weakens or the gland dries up. This results in the disappearance of pain and improvement of motor function or adiaphoresis. In rare cases, undesirable effects last as long as the beneficial effects, i.e., weeks to months. Eventually,

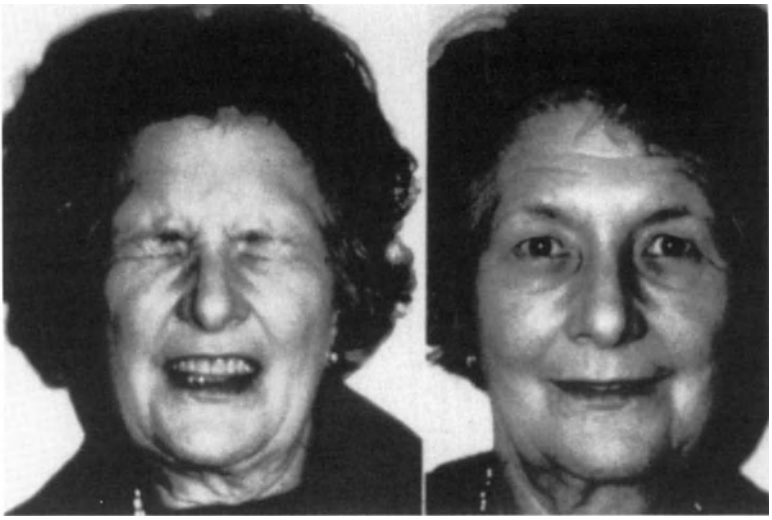

Fig. 8. Paralytic action of botulinum neurotoxin A complex in a patient suffering from blepharospasm. This patient exhibited sustained contractions of the periocular muscles, resulting in functional blindness (*left*). Nanogram amounts of the toxin inhibited acetylcholine release from nearby peripheral nerve endings, thereby relieving spasms (*right*)

nerve function recovers, and repeated treatments are required. Normally, BoNT/A does not stimulate the immune system, because the dose necessary to paralyze a muscle is much lower than that required for antibody production. In a small number of cases enough antibodies are produced to render the patient refractory to further treatment with BoNT/A. Patients with an anti-BoNT/A-antibody titer, however, do respond to other serotypes of BoNT, because different serotypes lack cross-immunogenicity. Recently, hemagglutinin complexes of BoNT/B, C_1 and F were found to be active in man (Sheean and Lees 1995; Green and Fahn 1996; Eleopra et al. 1997; Sloop et al. 1997). These serotypes, together with type A, should be made available for testing in the form of highly purified neurotoxins devoid of hemagglutinins and non-toxic, non-hemagglutinating proteins. The latter proteins are not necessary to protect neurotoxins against proteases during parenteral administration and have the added disadvantage of potentially causing unwanted reactions, including immune adjuvant effects and allergic responses.

The worldwide availability of highly potent toxins, especially of neurotoxins, has led to a growing concern about the possibility of misusing these agents as biological weapons. However, these fears appear to be largely unfounded, because, if dispersed into the environment, the activities of the toxins are very unreliable. If chlorinated domestic water supplies were to become contaminated with toxin, the latter would rapidly become deactivated by hypochlorite ions in the water. Deactivation also results from boiling drinking water. In addition, the potency of ingested clostridial neurotoxins is

decreased by a factor of 100000 as compared with parenteral administration (Ohishi et al. 1977). Therefore, there appears to be little cause for concern with regard to ingested purified toxin. Apart from the enteral route, other forms of delivery are highly impractical. High-molecular-weight proteins dispersed as aerosols are far less readily absorbed through the airways than low-molecular-weight nerve gas. In contrast to nerve gases, the residual potency of dispersed clostridial neurotoxins is not easily predicted. At least during the period of daylight, the large surface areas of aerosols ensure exposure of the toxin molecules to ultraviolet light, resulting in their inactivation. The purer the toxin preparation, the more unstable it is in the atmosphere and the less readily it is absorbed. Thus, the outcome of toxin contamination of air or water is equally unpredictable. Conceivably, a potential user might face an incalculable risk that far outweighs a possible hazard to an enemy. Consideration of all of these factors leads one to conclude that purified research toxins, while sophisticated and highly valued scientific tools, are too poorly absorbed and too unstable to be useful in warfare.

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CHAPTER 19

Anthrax Toxin

S.H. Leppla

A. Introduction

The virulence of *Bacillus anthracis* depends principally on two extracellular products, an antiphagocytic poly-γ-D-glutamic acid capsule, and a three-component protein exotoxin (Smith et al. 1955). The central role of these two virulence factors is demonstrated by the greatly reduced virulence of strains purged of either of two large plasmids, pXO1 and pXO2, which encode the toxin and capsule, respectively. Furthermore, the fact that antibodies to the toxin protect animals from infection (Gladstone 1946) demonstrates that anthrax, like diphtheria and tetanus, is a strongly toxin-dependent disease. Sterne-type *B. anthracis* strains lacking pXO2 are effective live vaccines, because they are avirulent but still able to make toxin and induce anti-toxin antibodies (Sterne 1937).

Anthrax toxin is unique in that it consists of three proteins which are individually non-toxic. The paradigm of the A-B toxins (Gill 1978) is modified, in the case of anthrax toxin, by having the binding and catalytic domains on separate proteins rather than within a single polypeptide. Furthermore, there are two alternate effector catalytic A moieties, so the term "anthrax toxin" is actually a collective term describing two separate biological activities. From the time when the multi-component nature of the toxin was first established, a major challenge has been to understand how the three proteins interact with each other and with cells to cause toxicity. Molecular descriptions of these interactions are now available and form a major theme of this chapter.

The multi-component nature of anthrax toxin helped to define a toxin-design paradigm to which more recently identified toxins conform. Several of these "binary toxins" have sequence similarity with anthrax toxin, implying the existence of a family of structurally related toxins. These toxins are discussed below to suggest that the molecular design exemplified by anthrax toxin has been horizontally transferred between several diverse pathogenic bacterial species.

The three proteins comprising the anthrax toxins are protective antigen (PA, 83 kDa), lethal factor (LF, 90 kDa), and edema factor (EF, 89 kDa). PA retains the name assigned to it when it was identified as the component in culture supernatants that confers protective immunity (Gladstone 1946). It is now recognized as the central receptor-binding component, which delivers

the two catalytic components into the cytosol of cells. Thus, the combination of PA + LF, named anthrax lethal toxin (LeTx), kills animals and certain cultured cells due to the intracellular delivery and action of LF, recently proven to be a metalloprotease (Duesbery et al. 1998; Vitale et al. 1998). The combination of PA + EF, named edema toxin (EdTx), disables phagocytes and (probably) other cells due to the intracellular adenylate-cyclase activity of EF (Leppla 1982). *B. anthracis* pathogenesis and anthrax toxin have been reviewed elsewhere (Leppla 1991b, 1995; Thorne 1993; Petosa and Liddington 1996; Friedlander and Brachman 1998; Hanna 1998; Quinn and Turnbull 1998).

B. Toxin Genes

I. Gene Location and Organization

The genes for all three toxin components are located on pXO1, the larger of the two plasmids present in virulent *B. anthracis* isolates. The genes for PA (*pagA*, previously named *pag*; see below), LF (*lef*), and EF (*cya*) are not contiguous but are spread over a 25-kb region of the 184-kb plasmid. A larger 40-kb region, which includes the toxin genes, is inverted in some strains of *B. anthracis* (Thorne 1993). The boundaries of the inverted region suggest it may be a mobile genetic element, perhaps one that can be considered a pathogenicity island. Only about five other genes have been identified on pXO1, so there remains a large amount of genetic information that could encode additional virulence factors. This situation will be clarified with the upcoming publication of the complete DNA sequences of pXO1 and pXO2.

II. DNA Sequences and Transcriptional Regulation

The DNA sequences of *pag*, *lef*, and *cya* of Sterne-type strains are available (Table 1). The structural gene for PA was recently renamed from *pag* to *pagA* when it was shown to be part of a larger transcriptional unit (Hoffmaster and Koehler, submitted). Because *pagA* from only one strain has been fully sequenced (Welkos et al. 1988), there is no evidence of toxin sequence variants. Little genetic variation would be expected, because amplified-fragment length-polymorphism analysis (Keim et al. 1997) shows *B. anthracis* to be a highly homogeneous species. Sequence determination of the three toxin genes identified ribosome binding sites and signal peptides preceding the genes encoding each of the three toxin components. Due to the low GC content of *B. anthracis* DNA, prediction of promoter sites is difficult.

Synthesis of both capsule and toxin are dependent on the presence of bicarbonate (Gladstone 1946; Strange and Thorne 1958; Bartkus and Leppla 1989). A gene required for bicarbonate-induced PA synthesis and gene transcription, initially identified by transposon mutagenesis, maps to the region of pXO1 between *cya* and *pagA* (Hornung and Thorne 1991; Thorne 1993).

Table 1. Anthrax-toxin-component genes and proteins

Toxin component	Protein properties			DNA sequence		Crystal structure	
	Amino acids in mature protein	Molecular weight ($\times 10^3$)	Calculated isoelectric point	Accession number	Reference	Accession number	Reference
PA	735	82.7	5.6	M22589	Welkos et al. 1988	1ACC	Petosa et al. 1997
LF	776	90.2	6.1	M29081; M30210	Bragg and Robertson 1989		
EF	767	88.8	6.8	M23179; M24074	Robertson et al. 1988; Escuyer et al. 1988		

This gene, *atxA* (Uchida et al. 1993; Koehler et al. 1994; Dai et al. 1995), also controls the transcription of *lef* and *cya*. Deletion analysis of the region upstream of *pagA* demonstrated that 111 bp of DNA upstream of the ATG start codon are sufficient to confer responsiveness to *atxA* (Dai et al. 1995). Transcriptional analysis of *pagA* identified two promoters. Promoter P2 is constitutively active at a low level, whereas, when *atxA* is present and bicarbonate is added, P1 produces messenger RNA amounts that are about tenfold above the constitutive amount (Koehler et al. 1994; Dai et al. 1995). Other work shows that a related gene on pXO2, *acpA*, is required for bicarbonate regulation of capsule synthesis (Vietri et al. 1995) and that there is a limited amount of cross-talk between *atxA* and *acpA* (Guignot et al. 1997; Uchida et al. 1997). There is preliminary evidence for a second transcriptional regulator, a putative repressor designated *atxR*, located near *lef* (Hornung and Thorne 1991; Leppla 1991b; Uchida et al. 1993). Transposon insertions at this locus make toxin synthesis constitutive, i.e., independent of bicarbonate. A simple model would have bicarbonate altering the repressor protein so that it is no longer able to block *atxA*-induced transcriptional activation.

C. Toxin-Component Proteins

I. Toxin Production, Purification, and Properties

Because PA is the active component of the licensed human anthrax vaccine, much effort was directed toward optimizing PA production and purification (Puziss et al. 1963). For vaccine production, anaerobic growth of Sterne-type strains in a defined medium yields 20–50 mg PA per liter, and lesser amounts of LF and EF (Leppla, 1991a). The LF and EF are largely removed by adsorption to porous glass filters during processing of the culture supernatant to produce the licensed U.S. vaccine. Purification of the toxin components for

research purposes can be accomplished by any of several standard column-chromatographic procedures, because the toxin proteins constitute the majority of the extracellular protein (Leppla 1988; Quinn et al. 1988).

Alternative methods for production of the toxin components are now available, including production from *Escherichia coli* and from protease-deficient *B. subtilis* and *B. anthracis* strains (Table 2). In the author's laboratory, PA and PA mutant proteins are produced (from the shuttle vector pYS5 in protease-deficient *B. anthracis* strains lacking pXO1 and pXO2) with yields of 30–50 mg per liter of culture (Singh et al. 1989). The pYS5 plasmid has 162 bp of pXO1 DNA upstream of the ATG start codon, preceded by a truncated bleomycin-resistance gene derived from the pUB110 plasmid. Because the *B. anthracis* strains used for PA expression lack pXO1 (and therefore *atxA*), we speculated that the bleomycin promoter was responsible for the high-level PA expression from pYS5. However, primer-extension analysis shows that the P1 promoter – the one normally requiring *atxA* activation – is active under the growth conditions used (rich FA medium with vigorous aeration; Park and Leppla, submitted). Methods for production of LF were recently improved by substituting its structural gene for that of PA in pYS5. The pro-LF molecule is expressed and secreted in an amount comparable to that of PA (i.e., 20–50 mg/l), and the PA signal peptide is properly cleaved to produce native LF (Park and Leppla, submitted).

The *B. anthracis* host used in the author's work on protein expression from pYS5 plasmids was derived from a plasmid-free indole auxotroph, UM44–1C9, by mutagenesis with transposon Tn917 and selection of a protease-deficient colony on casein–agar plates. The resulting strain, *B. anthracis* BH441, can be transformed by electroporation with unmethylated pYS5 plasmids to obtain effective producer strains. BH441 derivatives and other *B. anthracis* strains can be made sporulation deficient, as is preferred in order to limit possible spore contamination of laboratory areas. Detailed procedures were recently published for production of PA using plasmids similar to pYS5 in a sporulation-deficient *B. anthracis* strain grown in a rich medium similar to FA (Farchaus et al. 1998).

The three toxin proteins are water soluble in their native states and are stable during extended storage. PA and LF can be lyophilized, with full retention of activity. All three proteins lack cysteines and, therefore, disulfide bonds. PA and LF can be denatured and readily refolded with regain of activity (Leppla, unpublished). Inclusion of the constituent ions calcium and zinc is necessary for renaturation of PA and LF, respectively. The absence of cysteine also makes it convenient to add single cysteines as reactive sites for functional analyzes (Klimpel and Leppla 1996; Benson et al. 1998; Singh et al. 1999).

II. PA Structure and Function

The ready availability of purified PA made possible extensive studies of its structure and function. Two sites are uniquely sensitive to cleavage by

Table 2. Methods for toxin-component production and purification

Toxin component	Bacterial host	Expression plasmid	Promoter	Purification method[a]	Yield[b] (mg/l)	References
Protective antigen	*Bacillus anthracis*	pXO1	PA		40	Puziss et al. 1963; Leppla 1988; Leppla 1991a
	B. anthracis	pXO1	PA		0.8	Rogov et al. 1995
	B. anthracis	pXO1	PA			Pezard et al. 1993
	B. anthracis	pPA102	PA		30	Farchaus et al. 1998
	B. anthracis	pYS5	PA		40	Singh et al. 1989; Leppla 1991b
	B. subtilis	pPA102	PA			Ivins and Welkos 1986
	B. subtilis	pPA101	PA		7	Baillie et al. 1998; Miller et al. 1998
	Escherichia coli	pMS1	T7	PeriP	0.5	Sharma et al. 1996
	E. coli	pET22b	T7	PeriP	0.5	Benson et al. 1998; Roberts et al. 1998
	E. coli	pET15b	T7	His6	4	Willhite and Blanke 1998
Lethal factor	*B. anthracis*	pXO1	PA		10	Leppla 1988, 1991a
	B. anthracis	pYS5	PA		25	Park and Leppla, submitted
	B. anthracis	pXO1	LF			Pezard et al. 1993
	E. coli	pET15b	T7	His6	1.0	Roberts et al. 1998
	E. coli	pQE30	T5	His6	1.5	Gupta et al. 1998
	E. coli	pGEX-2TK	pTac	GST		Vitale et al. 1998
Edema factor	*B. anthracis*	pXO1	EF		10	Leppla 1991a
	B. anthracis	pXO1	EF			Pezard et al. 1993
	B. anthracis	pXO1	EF		0.5	Rogov et al. 1995
	E. coli	pUC8	Lac		2 (Cya62[c])	Labruyere et al. 1990

EF, edema factor; *LF*, lethal factor; *PA*, protective antigen.
[a] Purification was by standard chromatographic methods from culture supernatants (no entry) or from a periplasmic extract (PeriP) or by affinity chromatography on nickel-chelate columns (His6) or glutathione–agarose (GST).
[b] Yields, where reported, are the maximum amounts of purified protein reported by the authors.
[c] Truncated EF-containing amino acids 262–767, designated Cya62.

proteases. Specific cleavage at amino acids (aa) 164–167 (RKKR) occurs rapidly with 0.1 μg/ml trypsin (LEPPLA et al. 1988; NOVAK et al. 1992). Similarly, specific cleavage at aa 313–315 (FFD) occurs rapidly with 1.0 μg/ml chymotrypsin or thermolysin (LEPPLA 1991b; NOVAK et al. 1992; SINGH et al. 1994). The nicked PA proteins do not immediately dissociate into fragments. The 37-kDa and 47-kDa fragments produced by cleavage at aa 313–315 can be separated only after denaturation or treatment with certain detergents. Cleavage with both proteases produces three fragments, shown to be 19 kDa (aa 8–167), 17 kDa (aa 168–314), and 47 kDa (aa 315–735; NOVAK et al. 1992). These fragments were used to map monoclonal antibodies (LITTLE et al. 1988, 1996) and to identify functional domains (NOVAK et al. 1992), as discussed below.

The properties of the C-terminal 63-kDa fragment (PA63) produced by trypsin cleavage are especially informative. Chromatography of this preparation on the anion-exchange resin MonoQ produces a stable, heptameric species (LEPPLA 1991b) that can be visualized by electron microscopy (MILNE et al. 1994), non-denaturing gel electrophoresis (SINGH et al. 1994, 1999), or X-ray diffraction (PETOSA et al. 1997). This heptamer is toxic when combined with LF, demonstrating the dispensable nature of the N-terminal fragment, aa 1–167.

The crystal structure of PA, at 2.1-Å resolution, was solved by X-ray diffraction (Protein Data Bank accession code 1ACC; PETOSA et al. 1997; Fig. 1). PA is a tall, flat molecule with four distinct domains that can be associated with functions previously defined by biochemical analysis. The protein consists largely of β-structure, with only a small number of helices. Domain 1, aa 1–258, consists of two subdomains separated by a loop, aa 162–174. This loop is not resolved by X-ray diffraction, implying that it is flexible and solvent exposed, properties consistent with the ease of cleavage at aa 164–167 (RKKR) by proteases, such as trypsin and furin. Cleavage in this loop to produce PA63 is essential to toxicity. Cleavage at aa 164–167 allows release of the N-terminal 20-kDa fragment, thereby removing steric constraints and allowing oligomerization of PA63. Release of the 19-kDa fragment also exposes a new surface on domain 1′ (aa 168–258). It is to this surface that LF and EF are considered to bind. The newly exposed surface on domain 1′ overlays two buried calcium ions, each chelated by four acidic aa side chains. This structure is similar to the EF-hand motif characteristic of many calcium-binding proteins. The presence of calcium ions in PA had not been detected biochemically and was only discovered through the crystal-structure determination.

The surface region of domain 1′ exposed by removal of aa 1–167 is relatively hydrophobic. The details of the interaction of LF and EF with this region are not well understood. A monoclonal antibody, PA 1G3, generated by immunization with PA63, reacts with the 17-kDa fragment (aa 168–314) and blocks the binding of LF (LITTLE et al. 1996). This antibody does not react with intact PA, showing that removal of the 20-kDa fragment exposes the site to which the antibody and LF bind. The affinity of the antibody appears to be dependent on calcium, suggesting that it recognizes the surface overlaying the two chelated calcium ions (VARUGHESE and LEPPLA, unpublished work).

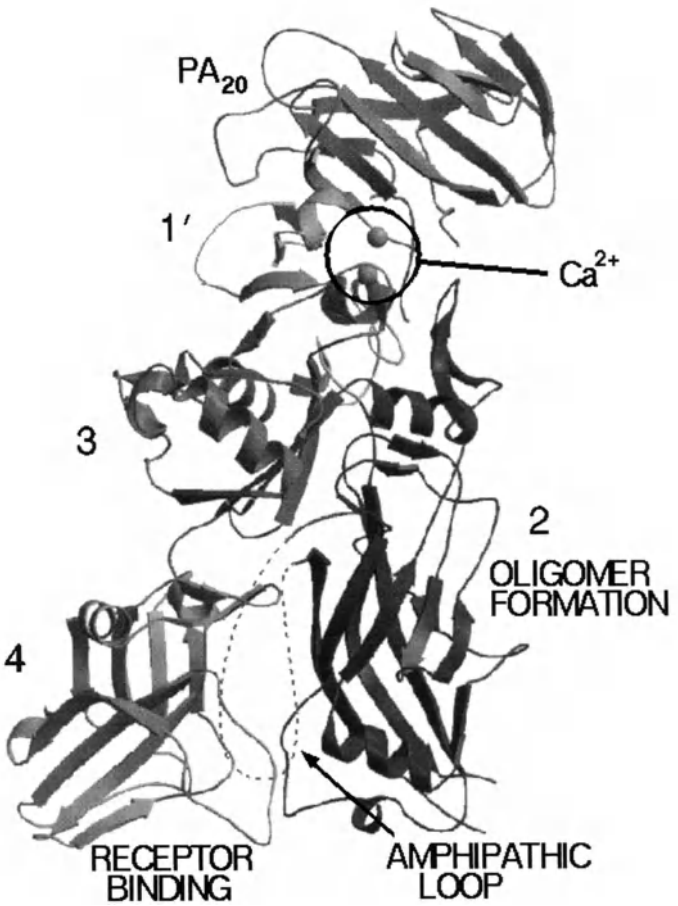

Fig. 1. Crystal structure of protective antigen at 2.1-Å resolution (Protein Data Bank accession code: 1ACC; Petosa et al. 1997). The domains are numbered consecutively from the N-terminus. Domain *1*: amino acids (aa) 1–258; domain *2*: aa 259–487; domain *3*: aa 488–595; domain *4*: aa 596–735

Domain 2, aa 259–487, is notable for several very long β-strands reaching nearly the full length of the domain. This domain forms the core of the heptameric assembly of PA63 subunits, which is considered to be the membrane channel through which LF and EF translocate. The most novel feature in domain 2 is the loop at aa 302–325. Like the loop at aa 162–174, this region was not resolved in the crystal structure. The sequence cleaved by chymotrypsin and thermolysin, aa 313–315 (FFD), lies in the center of this loop A pair of hydrophobic Phe residues would normally be buried inside a protein, so the sensitivity of this sequence to proteolytic cleavage is consistent with its exposure to solvent. By comparison to *Staphylococcus aureus* α-hemolysin, it was recognized that the loop at aa 302–325 has the alternating hydrophilic and

hydrophobic amino acids needed to form a β-hairpin (Petosa et al. 1997). In the staphylococcal α-hemolysin, seven such hairpins assemble into a β-barrel that forms the lytic pore. The β-barrel nature of the pore is discussed more fully below.

Another unique feature identified in domain 2 is a loop at the bottom surface, which contains several solvent-accessible hydrophobic aa. A sequence within this loop, aa 343–352 (ERTWAETMGL), was resolved in the structure determined at pH 7.5 but unresolved (and presumably more flexible) in the structure determined at pH 6.0. Because this region faces the target cell, it may play a role in the acid-dependent insertion of PA into membranes. Four of the five proteins with homology to PA (to be discussed below) have hydrophobic aa in the positions corresponding to W346, M350, and L352 and, therefore, could share any pH-dependent membrane-insertion event dependent on this region.

Domain 3, aa 488–595, is the smallest domain and the one for which the least functional information is available. Domain 4, aa 596–735, is the receptor-binding domain. This domain has limited contacts with other domains and could swing out of the way as the other domains forming the core of the PA63 heptamer insert into the endosomal membrane. The sequence homology of PA to other toxins now recognized as being in the same family does not extend into domain 4. This suggests that the receptor-binding domains of the PA family members may have been acquired from different sources. This could confer specificity against cell types and tissues found in the particular niches of the respective pathogens. In this context, it can be noted that the region upstream of PA on pXO1 contains a small gene that has strong sequence homology to PA domain 4 (Welkos et al. 1988; Leppla 1995). This may be a remnant of another receptor-binding domain.

Extensive mutagenesis has been done to characterize domain 4. Truncation of 3–7 aa from the C-terminus decreases both binding to receptor and toxicity about 20-fold. Larger truncations from the C-terminus destabilize the protein. Two loops at the bottom of PA, on the surface presumed to face the receptor, were analyzed by mutagenesis. Multiple substitutions or deletions in the larger loop, aa 704–723, decrease toxicity by at most tenfold (Brossier et al. 1999; Varughese et al. 1999). In contrast, certain substitutions in the smaller loop, aa 679–693, completely eliminate toxicity (Varughese et al. 1999). This demonstrates that the small loop is an essential part of the surface that interacts with the receptor.

III. LF Structure and Function

Good-quality crystals of LF have been obtained, and structural determination is underway. Until a crystal structure is available, knowledge of LF structure and function is limited to what is deduced from sequence comparisons, mutagenesis, and fusion proteins. LF and EF have substantial sequence homology in aa 1–250. Systematic deletion of LF fusion proteins containing the catalytic

domain of *Pseudomonas* exotoxin A established that LF aa 1–254 (LFn) are sufficient to achieve translocation to the cytosol of cells in a PA-dependent process (Arora et al. 1992; Arora and Leppla 1993). Deletion of aa 1–40 or 198–254 eliminated the translocation activity of LFn. Somewhat surprisingly, LFn functions equally well when "passenger" polypeptides are attached at either the N-or C-terminus (Arora and Leppla 1994a; Milne et al. 1995). LFn fusion proteins are being used to deliver a variety of polypeptides into cells, as discussed below. In the author's laboratory, a highly cytotoxic LFn fusion to the adenosine diphosphate (ADP)–ribosylation domain of *Pseudomonas* exotoxin A (FP59) is routinely used to measure the internalization process. FP59 kills any cell type that contains receptors for PA, whereas native LF is highly specific for macrophages. For this reason, FP59 has been useful in selecting toxin-resistant somatic cell mutants.

Adjacent to the LFn region are five imperfect repeats of 19 amino acids. Comparisons of the individual repeat sequences indicates they arose through several duplication events, suggesting that evolutionary pressure caused lengthening of this region. The overall repeat region, aa 256–383, is highly hydrophilic, with 55 of the 128 amino acids being charged. Sequence comparisons do not provide clues about the possible function of this region. Because there are few examples of repeated sequences within catalytic domains, this repeat region is believed to have a structural role, perhaps serving as a spacer domain to correctly position the adjacent catalytic domain.

Mutagenesis of LF has provided support allowing the two domains listed above to be distinguished from a putative C-terminal catalytic domain. Random insertions of di- and tetra-peptide sequences into LF showed that the LFn region is involved in binding to PA, whereas the C-terminal region is essential for toxicity but not required for PA binding (Quinn et al. 1991). The intermediate, repeat region is rather tolerant of insertions, although some insertions make the protein highly susceptible to proteolysis (Quinn et al. 1991). Substitutions of the nine His residues within aa 1–254 showed that only one of these plays a measurable role in activity. Thus, the H35G mutation decreased activity tenfold, whereas no other substitutions had an effect (Arora 1997).

The results discussed suggest that aa 350–767 contains the catalytic domain. Direct proof of this hypothesis began with the identification of a potential zinc-binding site at aa 686–690, which has the sequence HEFGH (Klimpel et al. 1994), consistent with the consensus HExxH sequence. Substitution at any of the residues in this sequence inactivates LF, and substitution of the His residues eliminates the zinc-binding ability of LF.

Recent work showed that LF is a highly specific metalloprotease (Duesbery et al. 1998; Hammond and Hanna 1998; Vitale et al. 1998). LF cleaves mitogen-activated protein kinase kinases 1 and 2 (MAPKK1, MAPKK2) very close to the N-terminus, thereby destroying their catalytic activity. The cleavage of the MAPKKs is observed both in vitro and in toxin-treated cells. The LF E687C mutant lacks protease activity, consistent with its lack of toxicity in cells. LF also cleaves certain peptides very slowly (Hammond

and Hanna 1998). The yeast two-hybrid system selected a MAPKK2 clone lacking aa 1–30, the region which contains the cleavage site (Vitale et al. 1998). This shows that LF binds to a site far from the bond that is cleaved, a property shared with the clostridial neurotoxins (Pellizzari et al. 1996; Cornille et al. 1997).

IV. EF Structure and Function

The N-terminal domain of EF (EFn) serves the same function as the corresponding domain of LF – translocation of the catalytic domain to the cytosol of cells. Thus, fusions of EF aa 1–254 to the catalytic domains of ADP-ribosylating toxins are highly cytotoxic, in the same way as FP59 (Arora, unpublished). Furthermore, monoclonal antibodies that block EF binding to PA63 have been mapped to EF aa 1–156 using the N-terminal fragment produced by acid cleavage at the Asp156–Pro157 bond (Little et al. 1994). EF is translocated less efficiently than LF from the cell surface in the acid-induced process that is used to model translocation from endosomes (Wesche et al. 1998). Although a lower affinity of EFn for PA63 could contribute to this difference, this system is also responsive to the properties of the catalytic or "passenger" polypeptides.

EF does not contain a repeat-containing domain like that in LF. Instead, the catalytic domain appears to begin soon after the EFn region. The catalytic domain lies entirely within aa 262–767, which was expressed in *E. coli* as a soluble, highly active adenylate cyclase, Cya62 (Labruyere et al. 1990, 1991). This truncated EF does not have the ability to enter cells, but it has been useful in characterizing the catalytic properties of EF. The enzyme has a K_{cat} of $1100\,s^{-1}$ and a K_m of 0.25 mM for adenosine triphosphate (ATP). Similar values for these parameters are observed with full-sized EF (Leppla 1984, 1991a).

The catalytic activity of EF, like that of the *Bordetella pertussis* adenylate cyclase, requires association with the eukaryotic, calcium-binding protein calmodulin. The dependence on calmodulin is absolute – no activity can be detected in its absence. Furthermore, no fragments of EF have been found that are calmodulin independent. This is unlike many calmodulin-dependent enzymes, where removal of a calmodulin-interacting domain produces a catalytically active species. Calmodulin is a much more potent activator of EF when it contains bound calcium. Thus, in the absence and presence of 50 μM calcium, the concentrations of calmodulin needed to activate EF are 5 μM and 2 nM, respectively.

Mutagenesis and comparison with the more extensively studied *B. pertussis* adenylate cyclase identifies aa 314–321 as the ATP-binding site, matching the GxxxxGKS consensus. The calmodulin-binding site extends over a large surface of the protein and probably includes aa 499–532, because a peptide with this sequence binds tightly to calmodulin (Munier et al. 1993).

The recent identification of the *exoY*-gene product of *P. aeruginosa* as an adenylate cyclase (Yahr et al. 1998) provides the third example, along with

EF and the *B. pertussis* adenylate cyclase (Chap. 10), of adenylate cyclases secreted by bacterial pathogens. Clearly, several diverse pathogens have captured the gene for an "invasive" adenylate cyclase and retained it for use as a virulence factor.

V. PA Family Members

Five toxin proteins that have sequence homology to PA are known (Table 3). Each of these PA relatives is the binding component of a binary toxin. In the four clostridial proteins, the separate catalytic component causes ADP–ribosylation of actin. The strong sequence homology to PA, which is confined to domains 1–3, argues that all these proteins will have a very similar overall structure and will form oligomeric channels that translocate the ADP–ribosylating component. The sequence conservation is perfect at the acidic amino acids shown to chelate calcium in PA, strongly suggesting that all these proteins will bind calcium. However, the proteolytic activation of the other proteins probably does not involve furin, because the putative cleavage sites do not include sequences easily cleaved by furin. The C-terminal domains of the proteins fall into three homology groups. The PA and the botulinum C2-toxin-receptor-binding domains have no homology to each other or to other proteins, whereas the other four proteins are closely related. The similarity of domains 1–3 of these six toxin proteins must be the result of the horizontal transfer of an ancestral gene to a diverse set of bacterial pathogens.

Table 3. Binary toxin components related to anthrax protective antigen

Protein toxin	Substrate for ADP–ribosylation	Length (residues)		Accession number	Reference
		Intact	Active fragment		
Bacillus anthracis protective antigen	NA	735	569	M22589	Welkos et al. 1988
B. cereus vegetative insecticidal protein	Unknown	884	664	Reserved	Warren et al. 1996
C. botulinum C2 toxin component II	Actin	721	540	D88982	Kimura et al. 1998
C. spiroforme toxin component Sb	Actin	879	669	X97969	Gibert et al. 1997
C. difficile binary-toxin component CDTb	Actin	876	665	L76081	Perelle et al. 1997a
C. perfringens iota-toxin component 1b	Actin	875	665	X73562	Perelle et al. 1993

ADP, adenosine diphosphate; *NA*, not applicable.

Clearly, this structural design has provided a base from which each pathogen developed a toxin that contributed to its virulence.

D. Cellular Uptake and Internalization

I. Cellular Receptor for PA

The receptor for anthrax toxin PA is not known. PA binding to the surfaces of cells has all the properties expected of a well-defined protein–receptor interaction. Binding is saturable and highly specific, with excess (unlabeled) toxin completing more than 90% of the total binding. The binding isotherms are linear, identifying a homogeneous population of receptors with a K_d of about 1 nM. These receptors are present on almost every type of cultured cell that has been examined, varying in number per cell from 2000 to 50000. CHO and RAW264.7 cells have 20000–30000 receptors per cell. In the author's unpublished work the two cell lines shown to have the most receptors, about 50000 per cell, are the L6 rat myoblast line (ATCC CRL-1458) and the LOX IMVI human melanoma cell line (WEINSTEIN et al. 1997). Glycosylation-defective CHO cell lines, such as Lec2 (DEUTSCHER et al. 1984), are as sensitive to anthrax toxin as the parental cell line. Chemical cross-linking methods were done to characterize the PA receptor. In one case, the results suggested the receptor has a mass of 85–90 kDa (ESCUYER and COLLIER 1991), whereas another study indicated a receptor with a mass of 22 kDa (FRIEDLANDER and RAZIUDDIN 1992).

The author's laboratory has selected many CHO cell mutants deficient in PA receptor. These have been obtained after either chemical or retroviral insertional mutagenesis. All the mutants obtained map to the same complementation group. The ease of obtaining PA-receptor mutants shows that the receptor gene locus is functionally hemizygous in some CHO lines. The availability of receptor-deficient mutants may make it possible to identify the receptor gene by genetic methods. In a similar approach, a CHO mutant that is resistant to the botulinum C2 toxin, apparently due to mutation of the receptor gene, was obtained (FRITZ et al. 1995). Although the receptor-binding domains of PA and the C2 toxin have no evident sequence homology, it is still possible that the toxins recognize the same or related protein receptors. Therefore, identifying the receptor for one of the PA family members (Table 3) may help to identify receptors for others.

II. Proteolytic Activation of PA

PA requires cleavage at residues 164–167 to acquire the ability to bind LF and EF and to oligomerize. Thus, deletion of the RKKR sequence (SINGH et al. 1989) or substitutions that eliminate all but one basic amino acid (KLIMPEL et al. 1992) eliminate PA activity on cells. A PA mutant protein with the sequence AAAR is non-toxic in cells but can be activated by treatment with low con-

centrations of trypsin. Mutation of the native cleavage site from RKKR to AARR makes the protein resistant to furin but not to other, unidentified host cell proteases, which appear to recognize a pair of basic amino acids (KLIMPEL et al. 1992; GORDON et al. 1995). Mutation of the native site to RAAR retains sensitivity to furin but eliminates sensitivity to the putative dibasic-cleavage enzyme. Therefore, this PA mutant protein is inactive in furin-deficient CHO cells.

Cleavage by furin is probably a rate-limiting step in toxin action (SINGH et al. 1999). When endocytosis is prevented by incubation at 5°C, cleavage requires several hours. There is very little furin on the cell surface, most of it being in the trans-Golgi region, from which a small amount cycles to the cell surface (MOLLOY et al. 1999). Because the PA receptor is not identified, it is not known whether furin is preferentially located near receptor-bound PA so as to facilitate cleavage. The slow rate of furin cleavage has implications for the kinetics of LF and EF uptake, which are discussed later.

Of the five toxin proteins that have sequence homology to PA, only two have sites that can be cleaved by furin. Therefore, these toxins must be activated by other host proteases or by bacterial proteases. The anaerobic clostridial pathogens usually grow in confined anatomical sites, which co-localizes secreted bacterial proteases with the toxin. It will be useful to characterize the proteolytic activation of these toxins.

III. LF and EF Binding to PA63

PA63 has affinity for LF and EF. This was originally demonstrated by co-immunoprecipitation and sedimentation equilibrium (LEPPLA 1991b). Subsequently, the interaction was demonstrated by gel electrophoresis, gel filtration under non-denaturing conditions, and in an enzyme-linked immunosorbent assay format (SINGH et al. 1999). Non-denaturing gel electrophoresis demonstrates the existence of complexes of the PA63 heptamer with LF. Titrating PA63 with increasing amounts of LF leads to the appearance of a ladder of bands of increasing size (i.e., decreasing mobility). Adding an equimolar amount of LF collapses the ladder into the slowest moving band, which is believed to contain seven LF molecules bound to the PA63 heptamer. The absence of any free LF under these non-equilibrium electrophoresis conditions suggests the binding is very strong, although it is also possible that the tight LF binding to the PA63 heptamer results from cooperative interactions between LF molecules. Direct measurement of LF binding to PA on cells yielded a K_d of 0.24 nM (NOVAK et al. 1992).

LF can bind to both monomeric and oligomeric PA63 (SINGH et al. 1999). LF binding to monomeric PA63 was demonstrated by attaching PA to a surface to prevent oligomerization. PA, tethered to a plastic surface, was nicked with trypsin to produce monomeric PA63, and this was shown to bind LF, proving that oligomerization is not a prerequisite for LF binding. The fact that LF can bind to either monomeric or oligomeric PA63 leaves open the

question of when oligomerization of PA63 occurs relative to the other steps in the process. The ability of surface-bound PA63 to form pores and to translocate LFn fusion proteins when acid pulsed (discussed below) suggests that a significant fraction of PA63 has already oligomerized on the cell surface.

IV. Endocytic Uptake

The entry of the anthrax toxin components into cells will be determined by the nature of the PA receptor. Until the receptor is identified, our understanding of the trafficking of the toxin will be based largely on pharmacological studies. These argue strongly that entry is by endocytosis (Table 4). Chemicals and drugs that prevent acidification of endosomes are very effec-

Table 4. Pharmacological agents blocking anthrax lethal toxin action in mouse macrophages

Agent	Possible intracellular target or action	Protective concentration	Reference
NH_4Cl	Endosome acidification	10 mM	Friedlander 1986; Gordon et al. 1988
Bafilomycin	Vacuolar proton pump; endosome acidification	0.05 μM	Menard et al. 1996a
Bestatin	Aminopeptidases	1 mM	Klimpel et al. 1994; Menard et al. 1996b
Phenylalaninamide	Ubiquitination	1 mM	Klimpel et al. 1994
Lactacytin	Proteasome	5 μM	Tang and Leppla 1999
Dithiothreitol	Reactive oxygen species	1 mM	Hanna et al. 1994
N-Acetyl-L-cysteine	Glutathione concentration	10 mM	Hanna et al. 1994
Leucine–CMK	Proteases	10 μM	Klimpel et al. 1994
Hydroxamate chelators	Metallopeptidases or leukotriene A4 hydrolase	0.1 mM	Menard et al. 1996b
Genistein	Tyrosine protein kinase	50 μM	Shin et al. 1999
Calyculin A	Protein phosphatases	0.02 μM	Lin et al. 1996
Quinacrine	Phospholipase A2	20 μM	Shin et al. 1999
Neomycin	Phospholipase C	25 mM	Bhatnagar et al. 1999

CMK, chloromethylketone.

tive blockers of anthrax-toxin action on cultured cells (Friedlander 1986; Gordon et al. 1988, 1989; Menard et al. 1996a). The effects of various pharmacological agents on anthrax-toxin action closely parallel those on diphtheria toxin action (Draper and Simon 1980; Sandvig and Olsnes 1981) and differ from those observed with certain toxins that undergo retrograde transport to the endoplasmic reticulum. This has been viewed as evidence that translocation of LF and EF occurs from an early-endocytic vesicle. The fact that translocation of surface-bound LF can be induced by brief acid shock (Friedlander 1986; Gordon et al. 1988; Wesche et al. 1998), exactly as occurs with diphtheria toxin (Sandvig and Olsnes 1981), supports the view that LF and EF can translocate from an early endosome. A kinetic analysis showing that LF exits from a late-endocytic compartment (Menard et al. 1996a) does not seem to have considered the slow and rate-limiting steps of PA cleavage and LF binding (Singh et al. 1999). Kinetic analyses do not clearly resolve the question of how fast toxins enter cells. Toxins preincubated with cells to allow nicking were reported to enter with a half-life of 3 min (Novak et al. 1992) or 30 min (Singh et al. 1999). The latter result was used to argue that the use of a slowly internalized receptor would allow time for PA to be both proteolytically activated and to capture LF or EF (Singh et al. 1999). Use of a slowing internalized receptor would extend the parallels to diphtheria toxin, which enters slowly, on a non-professional receptor (Almond and Eidels 1994).

V. Channel Formation

All bacterial toxins that act in the cytosol must cross cellular membranes. The membrane-crossing activity of anthrax toxin can be studied more easily than for many other toxins, because the soluble PA63 heptamer corresponds in some way to the membrane-inserted, protein-conducting channel. The soluble PA63 oligomer was first recognized during attempts to purify the fragments produced by limited trypsin digestion (Leppla et al. 1988; Leppla 1991b). Precipitation of the product and poor recoveries after chromatography suggested that PA63 was aggregating. Chromatography on the anion-exchange resin MonoQ at pH 9.0 yielded a soluble product. Non-denaturing gel electrophoresis showed the material made in this way to be a stable, monodisperse, high-molecular-weight species (Leppla 1991b; Singh et al. 1999). Electron microscopy showed that this preparation is heptameric (Milne et al. 1994).

The PA63 heptamer has channel-forming activity in artificial lipid membranes (Blaustein et al. 1989; Finkelstein 1994). Intact PA is unable to form channels, consistent with the view that only the proteolytically activated, oligomerization-competent species can produce channels. Channel formation requires acidification. The channel in artificial membranes is cation selective, voltage dependent, and is blocked by quaternary ammonium ions in a voltage-dependent manner. Comparison of the behavior of symmetric quaternary ammonium ions of different sizes suggests that the channel lumen has a

minimum diameter exceeding 11 Å (BLAUSTEIN et al. 1989). Studies in liposomes also demonstrate acid-dependent formation of ion-conductive channels (KOEHLER and COLLIER 1991).

PA63, but not PA, forms ion-conductive channels in the plasma membranes of cultured cells (MILNE and COLLIER 1993). Channels are formed when cells containing surface-bound PA63 are exposed to low pH, with pH 5.0 being optimal. Nicked PA bound to cells and either exposed to pH 5.0 or internalized at 37°C becomes oligomerized, as demonstrated by sodium dodecyl sulfate (SDS) gel electrophoresis. The oligomer formed in this way is stable on heating in SDS, unlike the PA63 heptamer made in vitro as described above. This suggests that there is a low-pH-induced transition between an oligomerized, receptor-bound, prepore species, and a membrane inserted, functional pore or channel.

A PA mutant protein lacking residues 313–315, PA-FFD, is non-toxic due to its inability to translocate LF and EF (SINGH et al. 1994). This mutant protein forms heptameric oligomers in vitro but fails to form the SDS-stable oligomers observed with native PA (SINGH et al. 1999), indicating that the stable oligomers represent the translocation-competent channel. Interestingly, the PA-FFD mutant appears to have a slightly lower affinity for receptors (LEPPLA, unpublished) and is unable to be inserted in asolectin vesicles (WANG et al. 1998). These data show that aa 313–315 are important for membrane insertion; this is consistent with the model to be described below.

The crystal structure of the PA63 heptamer made in vitro, the putative prepore, was solved at a resolution of 4.5 Å (PETOSA et al. 1997). The heptamer is a 160-Å-diameter ring with a height of 85 Å and a lumen that is about 20 Å at its most narrow. The heptamer does not have an obvious hydrophobic outer surface, as would be needed for it to be inserted into membranes. Instead, it was recognized that the large, flexible loop at aa 302–325 has the alternating hydrophobic and hydrophilic residues needed to form a β-hairpin. Seven such β-hairpins can assemble to make a β-barrel in which the hydrophobic residues face outward. The crystal structure of the membrane-inserted form of *S. aureus* α-hemolysin has such a structure (SONG et al. 1996). It is suggested that the insertion of the PA aa 302–325 loop could be accompanied by an unfolding of domain 2. In this way, β-strands 1–4 (aa 262–368) could make an extended structure that allows insertion of the heptameric β-barrel in order to cross the membrane bilayer (PETOSA et al. 1997).

Several studies support this model of membrane insertion. PA63 inserted into liposome membranes was labeled with a lipid-soluble, photoactivated reagent and then digested with proteases (WANG et al. 1998). The radiolabeled peptide identified as being membrane inserted began at Ala258 and extended for at least 24 aa. This supports the hypothesis that residues 262–368 constitute the membrane channel. Residues lining the channel were identified by Cys-scanning mutagenesis, in which every aa in the sequence 302–325 was individually replaced by Cys (BENSON et al. 1998). When the proteins were inserted into lipid membranes, the resulting channels were blocked by methanethio-

sulfonate ethyltrimethylammonium when the Cys replaced one of the hydrophilic residues of the β-hairpin, but not when it replaced the alternating hydrophobic residues. This result is convincing proof that the membrane channel has the proposed β-barrel structure like that in the staphylococcal α-hemolysin.

It is interesting to note that several of the clostridial-toxin proteins that are closely related to PA do not appear to require acidification for their cellular action (Perelle et al. 1997b). It would appear that these proteins have developed an alternative way to initiate membrane insertion and channel formation. This difference in acid dependence may be related to the location of His residues, which are often associated with pH-dependent transitions. All members of this group except PA contain a His–Ser or His–Thr sequence following the β-barrel-forming region, and only PA has His in the sequence that is part of the β-barrel. Comparison of the crystal structures of these proteins may explain why they differ from PA in this important way.

VI. Translocation and Cytosolic Trafficking

Few details are known about the process by which LF and EF translocate across membranes. Initiation of the translocation process is not understood. The fact that polypeptides fused to either end of LFn are translocated (Arora and Leppla 1994a; Milne et al. 1995; Wesche et al. 1998) shows that entry must begin with an internal hairpin structure. This may be analogous to the initial events in signal-peptide-dependent secretion. The lumen of the PA63 channel is rich in acidic residues (Petosa et al. 1997), so the interaction with LF and EF may involve ionic interactions. Short, basic polypeptides (especially oligo-lysine peptides) attached to proteins, such as diphtheria-toxin-fragment A, can cause their internalization in a PA63-dependent manner (Blanke et al. 1996), suggesting that basic regions on LF and EF play a role in translocation. However, the oligo-lysine-induced uptake may not accurately model LF and EF internalization, because LFn does not block this process.

It is probable that LF and EF partially unfold to pass through the lumen of the PA63 heptameric channel. Evidence for this comes from studies of a series of LFn fusion-protein constructs internalized from the cell surface of L6 cells by acid shock (Wesche et al. 1998). Fusions to the ADP–ribosylation domain of diphtheria toxin were prevented from translocating by introduction of a non-native disulfide bond. Reduction of the disulfide bond allowed translocation. Similarly, translocation of a LFn fusion with dihydrofolate reductase was blocked by addition of the tight-binding substrate methotrexate.

LF and EF have an ability to lyse liposomes in the absence of PA63 (Kochi et al. 1994). The lytic action of LF is accelerated by low pH. This result was interpreted to suggest that LF and EF may participate in the process of membrane translocation. However, the properties of the PA63 channel suggest that hydrophobic interactions of LF and EF may be important in the binding

of these components to the hydrophobic surface at the top of the PA63 heptamer.

The potency of LF and EF will depend on their stability in the cytosol. The stability of LFn fusion proteins depends on the identity of the N-terminal aa (Wesche et al. 1998), consistent with the N-end rule of Varshavsky (1997). Thus, a fusion protein having an N-terminal Met was stable after acid-shock-induced internalization, whereas a corresponding protein with an N-terminal Phe was unstable, having a half-life of less than 1 h. LF and EF have N-termini, Ala and Met, that are expected to confer moderate and high stability, respectively. For comparison, in Vero cells, diphtheria-toxin fragment-A chains with Ala and Met termini have half-lives of 3 h and 20 h, respectively (Falnes and Olsnes 1998). If EF is stable in the cytosol, then some other processes must explain the rapid fall in cyclic adenosine monophosphate (cAMP) concentration that occurs following removal of EdTx from CHO cells (Leppla 1982).

E. Intracellular Actions

I. EF Adenylate Cyclase

EdTx treatment increases cAMP concentrations in nearly all cells tested. Concentrations of cAMP obtained vary between cell types, from 2 μmol cAMP/mg protein to 2000 μmol cAMP/mg protein (Leppla 1984; Gordon et al. 1988, 1989). cAMP-dependent protein kinase is the only cellular component responsive to this second messenger, and it is already fully activated at rather low concentrations of cAMP. Therefore, the only other effect of EF is to deplete ATP concentrations. For these reasons, most cell types survive EdTx treatment and recover after its removal.

The apparent role of EdTx in anthrax infections is to incapacitate phagocytes during the early stages of infection. Polymorphonuclear cells treated with EdTx are less effective at phagocytosis of *B. anthracis* spores and at responding to phorbol-ester treatment (O'Brien et al. 1985). In human monocytes, EdTx induces interleukin-6 production and blocks lipopolysaccharide-induced tumor necrosis factor (TNF)-α production (Hoover et al. 1994). These effects closely paralleled those obtained with dibutyryl cAMP. Therefore, it follows that the effects of EdTx are mediated entirely by its ability to increase cellular cAMP concentrations. There have been relatively few studies of EdTx action, probably because the effects of EdTx are predictable. However, the effects of EdTx will differ from the long-lasting changes induced by cAMP-modulated agents, such as cholera toxin, because cAMP concentrations fall quickly following EF removal (Leppla 1982), as noted above.

Activation of cAMP-dependent protein kinase can have diverse effects in different cell types by modulating signal-transduction pathways involving protein phosphorylations. Because the only known action of LF is to affect

protein-kinase pathways (discussed below), it is probable that EF and LF may act synergistically in some cases.

The fact that EdTx does not kill cultured cells is consistent with its limited role in virulence. Strains of *B. anthracis* having the *cya* gene disrupted show only a tenfold increase in LD_{50}, an effect much smaller than the over 1000-fold increases caused by *pag* or *lef* disruption (Cataldi et al. 1990; Pezard et al. 1991, 1993).

II. LF Metalloprotease

LeTx is considered the dominant virulence factor of *B. anthracis*. Intravenous injection of saturating doses of LeTx into Fischer 344 rats causes death in 38 min (Beall et al. 1962; Ezzell et al. 1984). LeTx treatment kills mice and other animals more slowly and with symptoms matching those observed in animals dying of anthrax infection. Progress toward understanding how LeTx kills animals began with the demonstration that mouse and rat macrophages are uniquely sensitive to the toxin (Friedlander 1986). Primary macrophages and immortalized macrophage cell lines are lysed in approximately 120 min. The deaths of the cells appear to be necrotic rather than apoptotic, although apoptotic cells are seen at intermediate toxin concentrations when the protein-phosphatase inhibitor calyculin is added (Lin et al. 1996). Some other cell types show growth inhibition by LeTx (Leppla 1991b), but macrophages are unique in their rapid cytolytic response.

The RAW264.7 macrophage cell line is a convenient system for studying early changes following LeTx addition (Bhatnagar et al. 1989; Hanna et al. 1992, 1993; Milne et al. 1995). Increases in ion fluxes are evident at 45 min, followed by ATP depletion and release of superoxide at 60 min. Morphological changes are evident at 75 min, and lysis occurs at 90–120 min. The final stages of lysis can be slowed by depletion of extracellular calcium, addition of calcium-channel blockers, or osmotic protectants, suggesting that cell-membrane integrity is being lost. Depletion of macrophages by treatment with silica makes mice resistant to LeTx, and infusion of RAW264.7 cells restores sensitivity (Hanna et al. 1993). The release of cytokines upon lysis of macrophages may contribute to the death of the mice, because antibodies to interleukin-1 and TNF-α protect mice from LeTx action.

A diverse group of pharmacological agents protect RAW264.7 cells from LeTx action (Table 4). Protection by some antioxidants argues that the oxidative burst contributes to the deaths of the cells (Hanna et al. 1994). Further evidence that the oxidative burst plays a role is the resistance of the IC-21 macrophage cell line, which is unable to produce superoxide (Singh et al. 1989; Hanna et al. 1994). However, one specific oxidative product, nitrous oxide, does not appear to be involved in cell death (Shin et al. 1999). Other recent work suggests an involvement of phospholipases and protein phosphorylation in macrophage death (Bhatnagar et al. 1999; Varughese et al. 1999). However, these studies should be viewed cautiously because, in some cases,

the inhibitors were used at high concentrations, where their specificities are uncertain. It is also a concern that pharmacological studies do not easily distinguish between the early events resulting directly from LF action and the late, secondary, stress-induced responses characteristic of necrotic cells. In the ideal case, a protective agent targeting an early event would protect when added early (0–30 min) but not when added late (60–90 min). This is the pattern observed with bafilomycin, which specifically blocks escape of LF from endosomes to the cytosol (Menard et al. 1996a).

The finding that some protease inhibitors protect macrophages from LeTx (Klimpel et al. 1994) led to the hypothesis that LF might be acting on or through a proteolytic mechanism. This hypothesis helped direct attention to the presence, in LF, of a potential zinc-binding site in the C-terminal domain (Klimpel et al. 1994). As discussed above, mutagenesis of the HEFGH site supported the hypothesis that LF is a metalloprotease, leading eventually to the discovery that LF cleaves MAPKK1 and 2 (Duesbery et al. 1998) and peptides (Hammond and Hanna 1998). Cleavage of the MAPKKs was discovered independently, using a two-hybrid screen for LF-binding proteins (Vitale et al. 1998).

MAPKK1 may not be the only substrate of LF in the cytosol of macrophages. It is not evident how inhibition of MAPKK1 could lead to the rapid lysis of the cells, and work is needed to identify any additional substrates and to trace the events that lead to the rapid cell lysis. The fact that many different pharmacological agents protect cells from LeTx-induced lysis suggests that lysis may result from the simultaneous failure of several different homeostatic systems. Macrophages have various defenses against stress and may be able to repair damage to a single system. Perhaps LF initiates damage to several systems, so that the cumulative result exceeds the repair capacity of the cell. A genetic locus, *Ltx1*, which controls the sensitivity of mouse macrophages to LeTx, maps to mouse chromosome 11 (Roberts et al. 1998). Sensitivity is dominant, so the *Ltx1* allele from A/J mice confers resistance, while the allele from Balb/cJ mice confers sensitivity. Identification of the protein encoded at this locus will help to explain the lytic action of LeTx on mouse macrophages. However, this protein may be only one of many components involved in the pathway(s) leading to cell lysis.

F. Therapeutic Applications of LF Fusion Proteins

The ability of LFn to translocate heterologous polypeptides into the cytosol of cells is being exploited. Initial studies showed that fusions of LFn to the catalytic domains of other toxins were highly active (Arora et al. 1992, 1994b; Arora and Leppla 1993, 1994a; Milne et al. 1995). The catalytic domains of toxins that act in the cytosol of cells must translocate across membranes and might be expected to unfold and refold in a manner compatible with translocation by the PA63 pore. Direct analysis of a set of diverse LFn fusion

proteins using the acid-induced-internalization model showed that only some heterologous proteins can successfully translocate (WESCHE et al. 1998). Tightly folded proteins did not translocate.

The LFn delivery system is being developed as a way to generate cellular immunity. A cytotoxic T cell response occurs when antigenic peptides are generated in the cytosol, associate with major histocompatibility complex (MHC) class-I molecules, and are presented on the cell surface. LFn fusion proteins can deliver peptides and proteins to the cytosol to initiate this process (GOLETZ et al. 1997a). A protective cytotoxic T cell epitope from listeriolysin-O was fused to LFn. Mice injected with the LFn-peptide and PA were partially protected from infection by *Listeria monocytogenes* (BALLARD et al. 1996). More useful for vaccination would be to deliver a larger peptide or protein in order to provide epitopes presented by many different MHC haplotypes. Thus, delivery of a LFn-HIV gp120 fusion protein and processing via the proteasome led to presentation of a known V3-loop epitope (GOLETZ et al. 1997b). Peptides chemically conjugated to LFn are also efficiently delivered (BALLARD et al. 1998a, 1998b).

G. Summary and Future Prospects

Continued progress in defining the structure and function of the anthrax-toxin components can be expected in the next few years. Identification of cellular receptors for PA and the related binary toxins will do much to help explain their action on cells and in animals. Solving the LF and EF crystal structures will be immensely useful in understanding how LF and EF enter cells and express their catalytic effects. Finally, the rapidly developing knowledge about signal-transduction and cell-death pathways will provide the tools needed to deduce a molecular description of how LF kills macrophages and, by extension, anthrax-infected animals. This knowledge can be expected to lead to the rational design of toxin inhibitors that may be efficacious in the treatment of anthrax infection.

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CHAPTER 20

Adenylyl-Cyclase Toxin from *Bordetella pertussis*

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A. Introduction and Background

Bordetella pertussis produces a novel acylated adenylyl cyclase (AC) that is able to insert itself into the cytoplasmic membrane of eukaryotic target cells. In its interaction with target cells, the toxin rapidly (in seconds to minutes) creates a membrane perturbation that allows ion conductance. In the same time frame, the catalytic domain is delivered to the cell interior, where it produces supraphysiologic levels of cyclic adenosine monophosphate (cAMP) from endogenous (host) adenosine triphosphate (ATP). In a process that appears to be related to the transmembrane-pore formation, this AC toxin also causes lysis of erythrocytes and, through this activity, is the molecule responsible for hemolysis on blood–agar plates on which *Bordetella* species are growing. Production of AC toxin is regulated by the *Bordetella* two-component regulatory system, *bvg*, and it is a protective antigen in animal infections with *B. pertussis*. By virtue of its multiple activities, this interesting toxin is a useful prototype for study of the processes by which large exogenous proteins can interact with and traverse the membranes of nucleated target cells without causing permanent damage or death to the cell.

The historical background of the discovery and initial characterization of AC toxin was provided in several previous reviews of this toxin and will not be covered in detail here (MASURE et al. 1987; HANSKI 1989; MOCK and ULLMANN 1993; HEWLETT and MALONEY 1994). Recognition by WOLFF and COOK that a commercial pertussis vaccine contained AC enzymatic activity led to the purification of a fragment of AC toxin from the culture medium of *B. pertussis* (WOLFF and COOK 1973; HEWLETT and WOLFF 1976). Other novel features of this molecule include its extracytoplasmic location and its activation (up to 1000-fold increase in enzymatic activity) by the calcium-binding regulatory protein calmodulin (HEWLETT et al. 1976; BERKOWITZ et al. 1980). It was subsequently found that, unlike most calmodulin-regulatory functions, activation of the enzymatic activity of this AC could occur in the absence of Ca^{2+}, albeit at lower calmodulin potency (GREENLEE et al. 1982; KILHOFFER et al. 1983).

Despite the unusual aspects of this bacterial product (including its location and dependence on a host protein for high-level activity), there was no apparent "toxin" activity of the 70-kDa fragment first studied. In 1982,

however, Confer and Eaton demonstrated that urea extracts of *B. pertussis* were able to increase cAMP levels in alveolar macrophages and neutrophils and thereby profoundly inhibit their phagocytic and oxidative responses (Confer and Eaton 1982). This observation established AC from *Bordetella* species as a "toxin" and led to the investigation of this fascinating protein by a number of laboratories. This toxin is also produced by two other *Bordetella* species, *B. parapertussis* and *B. bronchiseptica*, but the majority of the characterization and study has been done with AC toxin from *B. pertussis* (Gueirard and Guiso 1993; Betsou et al. 1995).

B. Gene and Protein Structure

Cloning and sequencing of the AC toxin gene by Glaser et al. provided the data necessary to identify the holotoxin molecule, which is 177 kDa but which migrates on sodium dodecyl sulfate polyacrylamide-gel electrophoresis with an apparent molecular weight of over 200 kDa (Ladant et al. 1986; Glaser et al. 1988a; Hewlett et al. 1989; Rogel et al. 1989). Glaser et al. recognized that the operon consists of the structural gene *cya*A and associated genes *cya*B, *cya*D and *cya*E, which are involved in secretion of the toxin (Glaser et al. 1988b). Subsequently, Barry et al. discovered an additional gene, *cya*C, which is required for post-translational activation of the toxin, as will be described below (Barry et al. 1991). The entire operon has significant homology with members of the family of bacterial proteins termed the RTX (repeat in toxin) toxins, including *Escherichia coli* hemolysin, *Pasteurella haemolytica* leukotoxin and other, apparently non-toxic, proteins (Glaser et al. 1988b; Welch 1991, 1995; Coote 1992; Baumann et al. 1993; Thompson et al. 1993).

The toxin molecule possesses four major domains recognized on the basis of the DNA sequence: the catalytic domain, containing the active site for the enzymatic activity (amino acids 1–400), a hydrophobic region (amino acids 500–700), the glycine- and aspartic-acid-rich repeats (amino acids 1000–1600) and a C-terminal secretion signal (amino acids 1601–1706). In addition to the accessory genes for activation and secretion, the repeats in the distal half of the protein constitute the main feature the toxin has in common with the RTX family. These repeats are present in an alkaline protease from *Pseudomonas aeruginosa*; and in that molecule, the repeats form a series of unusual parallel β rolls (Baumann et al. 1993). The crystal structure of the *Pseudomonas* protease has been solved and reveals tight binding of calcium between two loops of each repeat. Recent study of AC toxin indicates that its repeats result in a comparable structure for calcium binding (Rhodes et al., unpublished data). AC toxin is a calcium-binding protein in which presence of the divalent cation elicits a conformational change apparently required for interaction with target cells (Hewlett et al. 1991; Rose et al. 1995). AC toxin has the largest number of repeats (38–41, depending on the degree of degeneracy tolerated in defining a unit) among the RTX proteins that have been identified; most have 10–20

(Glaser et al. 1988; Welch 1991; Coote 1992; Rose et al. 1995). Although enzymatic formation of cAMP from ATP is not dependent on the presence of Ca^{2+}, other activities of the toxin, namely binding to target cells and cell-invasive and pore-forming/hemolytic functions, do not occur when no Ca^{2+} is available to the toxin (Confer et al. 1984; Hanski and Farfel 1985; Gentile et al. 1990).

As with some of the RTX toxins that have been studied in greater detail, non-enzymatic activities of AC toxin require the product of an ancillary gene, *cya*C, which is somehow involved in (presumed to be responsible for) an ε-amino, post-translational acylation of a lysine (Barry et al. 1991; Issartel et al. 1991; Hackett et al. 1994; Gray et al. 1998; Stanley et al. 1998). Unexpectedly, there is a difference in the acylation pattern between wild-type AC toxin expressed in the parent organism, *B. pertussis*, and recombinant AC toxin expressed in *E. coli*, with palmitoylation of the wild-type molecule at a single site (lysine 983) but mixed palmitoylation (approximately 67%) and myristoylation (33%) at lysine 983 plus partial palmitoylation at a second site (lysine 860) in the recombinant toxin, when expressed in *E. coli* XL-1 Blue (Hackett et al. 1995). These two preparations appear to have functional differences in their hemolytic activities but have comparable cell invasive activities (Hackett et al. 1995). The bases for the differential acylation and any functional differences remain unknown.

C. Biological Activities of AC Toxin

As indicated above, there are several functions of AC toxin that must be distinguished prior to considering the mechanism of action and role of this virulence factor in the diseases caused by *Bordetella* species. Development of a genetic construct by Sebo et al. enabling high-level expression of AC toxin in *E. coli*, was an essential step in the subsequent studies of toxin activities described below (Sebo et al. 1991).

I. Enzymatic Activity

The conversion of ATP into cAMP in an in vitro system consisting of AC toxin and substrate (Mg-ATP), with or without calmodulin, defines the enzymatic activity. This reaction can be catalyzed (with high turnover number and specific activity) by the holotoxin and virtually any of the proteolytic fragments of the toxin containing the catalytic domain. As with other cell-invasive toxins that are enzymes and conform to the A/B model, the isolated catalytic domain retains its enzymatic activity but has no "toxin" activity (Gill 1978; Bellalou et al. 1990). The structure of the catalytic domain and its interaction with calmodulin have been studied extensively (Haiech et al. 1988; Ladant 1988; Glaser et al. 1989; Ladant et al. 1989; Gilles et al. 1990; Bouhss et al. 1993; Munier et al. 1993). Among the observations was the demonstration by

Ladant that two tryptic fragments of the catalytic domain (T18 and T25) could be stabilized by calmodulin, protecting its enzymatic activity from proteolytic digestion whereas, in the absence of calmodulin, the activity is quickly lost (Ladant 1988). The ability to reconstitute the enzymatic activity has served as the basis for use of AC fragments in a two-hybrid system for evaluation of signal-transduction pathways in eukaryotic cells by Karimova et al. (1998).

II. Cell-Invasive Activity

The ability of AC toxin to increase the intracellular cAMP levels of intact cells results from the delivery of the catalytic domain and is defined as "cell-invasive" activity or "intoxication". Unlike some other bacterial toxins, the delivery process occurs from the cell surface without involvement of receptor-mediated endocytotic pathways (Hanski and Farfel 1985; Gentile et al. 1988; Gordon et al. 1988; Donovan and Storm 1990). The invasive step has also been quantified by determination of enzymatic activity in a trypsin-protection experiment (Bellalou et al. 1990; Rogel and Hanski 1992, Hewlett et al. 1993). Enzymatic activity in the lysate of cells exposed to toxin and then trypsin before lysis is compared with enzymatic activity contained in lysates in the absence of trypsin treatment (a reflection of the total amount of AC toxin bound). The results allow determination of the proportion of bound catalytic domain that has been delivered to a trypsin-resistant site, presumably the cytosol. Ca^{2+} is required for invasive activity, and there is a steep calcium-concentration dependence, with activity increasing from essentially none to full effect between 200 μM and 500 μM Ca^{2+} (Hewlett et al. 1991; Rogel and Hanski 1992). The delivery of the catalytic domain to the cell interior (but not the initial binding of the toxin) is dependent on the membrane potential of the target cell, with invasion blocked when the target cell is depolarized (Otero et al. 1995). Invasive activity is also temperature dependent, with little or no cAMP accumulation at 0–4°C (Rogel and Hanski 1992; Gray et al. 1998).

The stoichiometry of invasive activity suggests that it is a function of the toxin monomer (Gray et al. 1998). There are, however, data to suggest that AC toxin is able to interact with itself to form dimers or higher-order forms in the setting of invasive activity. First, Iwaki et al. constructed mutants from which the major domains of the toxin were deleted and used those molecules (alone and in pairs) to evaluate the consequences of the deletions for invasive activity (Iwaki et al. 1995). The results revealed that several mutants, which were completely inactive individually, were able to produce low-level cAMP accumulation in sheep red blood cells (RBCs) when added in combination. indicating that dimers must be formed to allow this complementation. Additional data are provided by recent work from Bejerano et al.. who showed that similar deletion mutants can be complemented by small peptides representing the critical areas that had been deleted (Bejerano et al. 1998). Especially important was a stretch of amino acids near the C-terminus, a domain

that had been identified previously as critical to toxin function (Iwaki et al. 1995). In addition to allowing identification of essential domains, the preparation of deletion mutants has been especially useful for mapping monoclonal antibodies against AC toxin, for which it had previously been impossible to identify epitopes except by homology with *E. coli* hemolysin (Lee et al. 1999).

III. Pore Formation and Hemolysis

In a process that is completely dissociable from cell invasive activity and independent of cAMP production, AC toxin is able to cause lysis of erythrocytes (RBCs) (Weiss et al. 1983; Ehrmann et al. 1991; Rogel et al. 1991; Sakamoto et al. 1992). This action of the molecule is responsible for the hemolytic phenotype of wild-type *B. pertussis* on blood–agar plates. Hemolysis elicited by AC toxin is rather slow and modest compared with that produced by other members of the RTX family but is, as with invasive activity, dependent on acylation and Ca^{2+} (Ehrmann et al. 1991). Although there are descriptions of AC toxin producing hemolysis in the presence of Ca^{2+} chelators, these results are interpreted as reflecting the persistence of toxin-bound Ca^{2+}, which cannot be removed without denaturation of the molecule (Rogel et al. 1991; Rose et al. 1995; Gray et al. 1998). These few calcium ions bound to AC toxin are believed to form a conformation of the toxin that allows binding to the target cell and development of hemolysis but is insufficient for invasive activity (Fig. 1).

The stoichiometry of hemolysis, as determined by concentration dependence, suggests that a higher-order form, such as an oligomer, is required (Szabo et al. 1994; Gray et al. 1998). Hemolysis has several other peculiar features, including some target cell specificity. For example, sheep, rabbit and mouse RBCs are sensitive to AC-toxin-induced hemolysis, while human RBCs are resistant, being susceptible to hemolysis only when the human erythrocytes are treated with *N*-ethylmaleimide prior to addition of AC toxin (Rogel et al. 1991; Gray et al. 1998). The basis for the differences seen with human RBCs is not known, but it does not appear to be in toxin binding, since cell invasion by the catalytic domain, resulting in cAMP accumulation, occurs without special manipulation of the conditions (Rogel et al. 1991; Gray et al. 1998).

Hemolysis is variable among preparations of AC toxin and, at toxin concentrations less than 10 μg/ml, the process exhibits a lag of greater than 1 h (Ehrmann et al. 1991; Rogel et al. 1991; Gray et al. 1998). As a result of these characteristics, identification of an early step in the sequence of events leading to hemoglobin accumulation in the medium has long been a goal. Several studies have been conducted using artificial lipid bilayers to investigate the ability of AC toxin to create transmembrane pores (Benz et al. 1994; Szabo et al. 1994). Szabo et al. demonstrated that ion conductance elicited by AC toxin is voltage-dependent, calcium-dependent and develops with a preference for negatively charged phospholipids (Szabo et al. 1994). Benz et al. observed

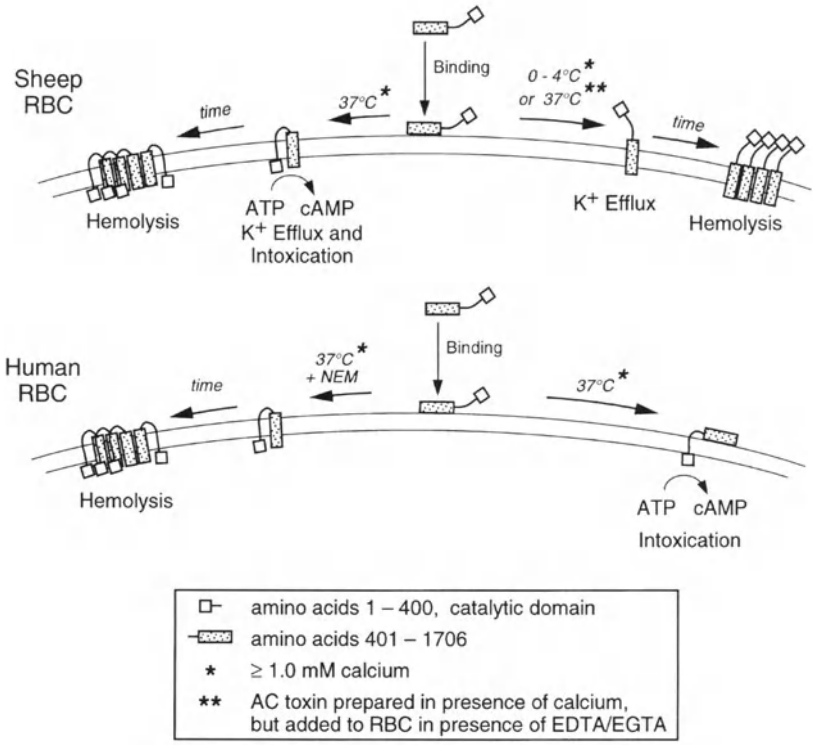

Fig. 1. Diagrammatic representation of the functional activities of adenylyl-cyclase toxin

cation-selective single channels with pore sizes smaller than that of *E. coli* hemolysin (Benz et al. 1994).

More recently, in pursuit of an easily measurable indicator of membrane insertion/perturbation by AC toxin, Gray et al. (1998) found a concentration-dependent K^+ efflux from target RBCs exposed to AC toxin. This process occurred with an onset (within minutes) and time course comparable to that of cell invasion but could be completely dissociated from delivery of the catalytic domain and the resultant accumulation of cAMP. The efflux of K^+ is attributed to creation of a defect in the RBC membrane by insertion of monomeric toxin, because the stoichiometry indicates a power dependence near unity. Although reduced temperature causes a marked delay in hemolysis but only a modest effect on K^+ efflux, the two processes are not dissociable, suggesting that K^+ efflux may represent an antecedent step to oligomerization and hemolysis. K^+ efflux was not observed in human RBCs under standard conditions that support K^+ efflux and hemolysis of sheep

RBCs. Together, these data can be interpreted as indicating that insertion of AC toxin to deliver its catalytic domain and insertion to allow K^+ release and hemolysis may occur by structurally different paths. In fact, at this time, it is not possible to determine whether both activities can actually be carried out simultaneously by the same toxin molecule.

IV. Summary

The generally accepted concept of AC toxin action begins with binding of the holotoxin molecule to the surfaces of target cells by interaction with an unknown host factor. This initial interaction is not saturable up to concentrations of toxin far in excess of those required to reach maximal levels of cAMP. There is then an insertion of some part of the toxin into the membrane, resulting in efflux of intracellular K^+. In erythrocytes, this process continues inevitably to hemolysis, presumably via steps including oligomerization of several toxin molecules to a higher-order form. Within the same time frame, there is delivery of the catalytic domain, which interacts with intracellular calmodulin to increase its enzymatic activity and produce cAMP. This complex sequence of events and the alterations imposed by different conditions and effectors are illustrated diagramatically in Fig. 1. The issue of whether both invasion and K^+ efflux/hemolysis can be carried out by the same molecule is not addressed in this model.

It has been proposed by Oldenburg et al. that the binding of calmodulin to its site on the catalytic domain is required for retention of the enzymatic portion of the molecule within the cytosol (Oldenberg et al. 1992). This concept has been disputed, however, on the basis of mutants with reduced affinity for calmodulin that were, nevertheless, capable of efficient invasiveness (Heveker and Ladant 1997). Storm and coworkers have also proposed an alternative hypothesis to the model presented above. The mechanism they propose involves the proteolytic cleavage of AC toxin into an enzymatically active 45-kDa fragment and one or more other fragments that facilitate the entry of the catalytic domain into the cell (Donovan et al. 1989; Oldenburg and Storm 1993). These two components would comprise the A and B subunits of the toxin in the A/B model of Gill, and neither would have activity in the absence of the other (Gill 1978). The peptide that facilitates entry is described as binding to wheat-germ lectin–agarose and being protease sensitive (Donovan et al. 1989). The predominant proteins eluted from the lectin column range in size from 26 kDa to 30 kDa. A synthetic peptide corresponding to amino acids 313–339 in the catalytic domain inhibits the invasive activity of a mixture of these fractions, suggesting that the interaction between the A and B subunits is disrupted in the presence of the peptide (Oldenburg and Storm 1993). This scenario, which does not deal with the issues of pore formation and hemolysis, has yet to be confirmed or supported by data from other laboratories. Although the majority of data indicate that AC toxin can act as

a single protein, meaning that the highest-potency preparations are those containing the greatest quantity of holotoxin, one cannot exclude the possibility that both models are correct. Additional studies designed specifically to address this possibility would be required to resolve the issue.

D. Possible Role/s of AC Toxin in Pathogenesis

Discovery of the invasive activity of AC toxin by Confer and Eaton resulted from experiments in which neutrophils and alveolar macrophages were exposed to extracts of *B. pertussis* (Confer and Eaton 1982). The effect on these cell types was a profound inhibition of their oxidative response to soluble and particulate stimuli and was correlated with the levels of intracellular cAMP. Several studies have shown that AC toxin is a relevant virulence factor for *B. pertussis* and that organisms defective in its production are reduced in ability to cause and sustain infection in experimental animals (Weiss et al. 1984; Brezin et al. 1987; Guiso et al. 1989; Weiss and Goodwin 1989; Khelef et al. 1992). This information and subsequent studies characterizing this phenomenon with different preparations of AC toxin led to the hypothesis that the toxin's function for the bacterium is to suppress the host's defense mechanisms for microbe clearance (Confer and Eaton 1982; Friedman et al. 1987; Pearson et al. 1987). This suggestion is consistent with the demonstration of the ability of intact *B. pertussis* organisms to deliver AC toxin to target cells by contact (Mouallem et al. 1990). There is no evidence that the toxin accumulates significantly in the environment or that the toxin or the organism circulates to sites outside of the respiratory tract to cause systemic effects during infection.

AC toxin has also been shown to cause apoptosis in some cells with which it interacts, especially alveolar macrophages (Khelef et al. 1993, 1995; Gueirard et al. 1997). The catalytic domain is required for this function, but the mechanism by which apoptosis is promoted is unknown. Since, in some infectious processes, apoptosis contributes to induction of inflammation, and AC-toxin-deficient mutants elicit less inflammation in animal studies, it is possible that this is a role of AC toxin acting through the induction of apoptosis (Gueirard et al. 1997; Zychlinsky and Sansonetti 1997). The other alternative is that the induction of cell death by this pathway is merely a mechanism for the creation of local damage to reduce host clearance processes.

B. pertussis and other *Bordetella* species have been observed to invade and survive, at least for short periods of time, in both phagocytic and non-phagocytic cells (Ewanowich et al. 1989a, 1989b; Steed et al. 1991; Friedman et al. 1992). Interestingly, the expression of AC toxin appears to be detrimental to invasion of non-phagocytic cells, such as HeLa cells, but beneficial for invasion of and survival within macrophages (Ewanowich et al. 1982a; Friedman et al. 1992). The potential clinical relevance of these in vitro studies

is supported by the observation by BROMBERG et al. (1991) that human immunodeficiency virus-infected children with pertussis exhibit *B. pertussis* organisms associated with alveolar macrophages.

In general, accumulated data indicate that AC toxin acts as a local toxin but, even within that concept, there is the possibility that it may contribute to the disease process in additional ways. As in the intestinal tract, the epithelium of the respiratory tract can be stimulated to secrete mucous and fluid by increases in intracellular cAMP concentration (BOUCHER 1994). Although there are no pathogens known to be comparable to *Vibrio cholerae* for the respiratory tract, it is well known that patients with pertussis have voluminous secretions, which are expectorated during cough paroxysms (LAPIN 1943). Since *B. pertussis* organisms are restricted to the respiratory mucosa or submucosa during infection and can deliver AC toxin to cells with which they come in contact, it is certainly plausible that cell invasion by this potent virulence factor could be responsible for a cAMP-mediated secretory response to account for these clinical findings. This possibility remains to be tested experimentally in systems that have been developed for investigation of the respiratory epithelium.

E. AC Toxin as a Protective Antigen

Humans infected with *B. pertussis* or immunized with whole-cell pertussis vaccines develop antibodies against AC toxin (FARFEL et al. 1990; GUISO et al. 1990; ARCINIEGA et al. 1991, 1993). There is, however, no information in humans with regard to possible protective activity of this immune response. GUISO and associates have presented, in a series of papers, the protective effect of AC toxin against infection of mice with *B. pertussis* (BREZIN et al. 1987; GUISO et al. 1989, 1991; BETSOU et al. 1993, 1995). In early studies, it appeared that only the catalytic domain was able to elicit protection in a mouse respiratory-infection model but, subsequently, it was determined that the holotoxin is required and that its post-translational acylation is also important (BETSOU et al. 1993). In that study, the time course of infection in animals receiving non-acylated AC toxin was the same as that in controls, whereas immunization with wild-type toxin elicited the most rapid clearance. Immunization with recombinant toxin was associated with an intermediate rate of clearance, providing the first suggestion that expression of AC toxin in *E. coli* could result in a different state of acylation.

Due primarily to the fact that it was not available in sufficient quantity and purity at the time of vaccine development, AC toxin was not considered seriously for inclusion in the acellular pertussis vaccines, which have recently been evaluated and licensed for use in routine pertussis immunization (HEWLETT and CHERRY 1997). Whether addition of this molecule (which is an established protective antigen in animals) to acellular vaccines for humans is beneficial remains to be determined.

F. Uses of AC Toxin as a Novel Research Reagent

Unlike the modest effects of cholera toxin on cAMP production in target cells, AC toxin from *B. pertussis* allows for extraordinary increases in cAMP to supraphysiologic levels, often several orders of magnitude above those achieved by physiologic agonists. The ability to make rapid, concentration-dependent alterations in this important intracellular mediator in the absence of effects on G proteins has been useful for investigation of cAMP-modulated processes in multiple cell types, including anterior pituitary cells, ovarian cells and cancer cells (Slungaard et al. 1983; Cronin et al. 1986; Veldhuis et al. 1988). For such studies, an enzymatically inactive mutant produced by a single amino acid substitution at lysine 58 serves as a control for any potential effects of the pore-forming domain (Au et al. 1989; Ehrmann et al. 1992).

Sory and Cornelis have used a chimera of the catalytic domain of AC toxin fused to YopE to demonstrate the entry of the *Yersinia* protein into the cytosol of target cells (Sory and Cornelis. 1994). In this creative application, the intracellular cAMP produced by the catalytic portion of the fusion protein serves as a marker of the delivery process.

More recently, Sebo et al. have demonstrated that a foreign epitope consisting of a 15-amino-acid sequence from lymphocytic choriomeningitis virus can be introduced into permissive sites within the catalytic domain of AC toxin without compromising the invasive activity of that domain (Sebo et al. 1995). Furthermore, the foreign epitope is subsequently presented on the target cell surface, causing the cell to be sensitized to CD8+ cytotoxic T-cells (CTL) directed against that peptide, indicating that it has been delivered to the cytoplasm and processed through the major histocompatibility complex class-I pathway. That this phenomenon is not just an in vitro artifact is illustrated by the ability of an intraperitoneally injected construct of AC toxin containing the foreign epitope to elicit antigen-specific CTL (Fayolle et al. 1996). These exciting results provide an opportunity for new approaches to immunization through the class-I pathway, which is otherwise difficult for exogenously delivered antigens to access and leaves open the possibility of introducing proteins into the cytoplasm for other purposes.

The genetic manipulation of the AC-toxin molecule has been further exploited by introduction of charge differences in the region of the epitope to be delivered (Karimova et al. 1998). The results (that additional positive charges impair entry of the domain) confirmed an earlier study by Otero et al. (1995) showing that invasive activity of AC toxin in single, patch-clamped frog heart cells is blocked by depolarization of the cell. Together, these data strongly support the concept that the invasiveness of the catalytic domain is driven, at least in some cells, by the transmembrane potential.

G. Future Directions

It should be apparent from this presentation that there are numerous basic issues that remain unresolved concerning AC toxin. These include: (1) identi-

fication of the molecule or molecules on the surfaces of cells that serve as targets for the first interaction with the toxin; (2) determination of the exact role of calcium and acylation in binding and subsequent steps of toxin action; (3) elucidation of the basis for differential acylation between wild-type toxin produced in *B. pertussis* and recombinant toxins expressed in different strains of *E. coli*; (4) examination of the mechanism by which the catalytic domain enters the cytoplasm and further characterization of the role of membrane potential in that process; and (5) elucidation of the structures formed by the glycine/aspartate-rich repeats when they bind calcium. In addition, continued use of AC toxin coupled with foreign peptides will provide novel approaches to examination of the consequences and localization of their delivery.

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CHAPTER 21

Helicobacter Pylori Vacuolating Cytotoxin

W. Fischer and R. Haas

A. Introduction

Helicobacter pylori is a gram-negative, strongly motile and spiral-shaped bacterial pathogen found in the stomach mucosa of more than 50% of the world population. The infection is usually acquired in childhood but persists for years and decades when untreated. A consequence of the infection is the induction of an inflammatory response, which is manifested histologically as chronic superficial gastritis. Although most infections remain asymptomatic, approximately 10% of infected people become sick, suffering from gastroduodenal ulcerations, gastric adenocarcinoma or mucosa-associated-lymphoid-tissue lymphoma.

Several factors have been shown to be essential for bacterial colonisation of the stomach mucosa. These include a potent urease (Labigne et al. 1991) allowing the pathogen to survive gastric acidity. By hydrolysing urea and producing ammonia, the bacteria are able to adapt their internal (cytoplasmic/periplasmic) and/or the external pH to survive under varying gastric pH conditions (Scott et al. 1998). The flagella confer strong motility to the mucus-adapted bacteria, allowing them to spread in the gastric mucin layer and to reach the epithelial surface. Bacterial outer-membrane adhesins are involved in specific binding of the pathogen to cell-surface receptors. Cross-talk is induced by signal-transduction events (tyrosine phosphorylation) between bacteria and eucaryotic cells (Borén et al. 1993; Segal et al. 1996; Ilver et al. 1998).

Like many bacterial pathogens, *H. pylori* produces and secretes a potent proteinaceous toxin, the vacuolating cytotoxin VacA. An immunodominant 128-kDa protein is found either on the surface or in the bacterial supernatant, but its expression correlates with the vacuolating activity and, thus, was termed CagA (cytotoxin-associated antigen A). VacA induces the formation of acidic vacuoles (Cover and Blaser 1992), leading finally to destruction of epithelial cells, whereas the function of CagA is still unknown. Interestingly, *cagA* is located on the border of a pathogenicity island (PAI), a 40-kb DNA fragment only present in the genomes of a subset of *H. pylori* strains. *H. pylori* strains producing CagA and VacA have been termed type-I strains, whereas those lacking PAI and VacA activity are type-II strains (Telford et al. 1994a). Type-I strains are considered more pathogenic since they are associated with the severe diseases like ulcerations and adenocarcinoma.

The *cag* PAI apparently encodes a type-IV secretion system. Such a system for the export of macromolecules has been identified in the plant pathogen *Agrobacterium tumefaciens* and the human pathogen *Bordetella pertussis*. *A. tumefaciens* exports its oncogenic T-DNA to plants, and *B. pertussis* uses the system for the export of its important virulence factor, pertussis toxin. The nature and functions of the molecules exported by *cag* PAI of *H. pylori* are still unknown. This review focuses on the vacuolating cytotoxin, a new representative of a bacterial AB toxin, its clinical relevance, its gene structure, aspects of gene regulation, secretion, structural and functional aspects of the extracellular cytotoxin and its interaction with epithelial cells.

B. Identification and Purification of *H. pylori* Vacuolating Cytotoxin

Cytotoxic activity in broth-culture filtrates of *H. pylori* was originally described by Leunk and co-workers (1988). They discovered that about 55% of *H. pylori* isolates from four geographic regions worldwide produced a proteinaceous cytotoxin that induced vacuolation in seven of nine eucaryotic cell lines tested. The effect of cytotoxin on HeLa cells in culture is shown in Fig. 1. In 1992,

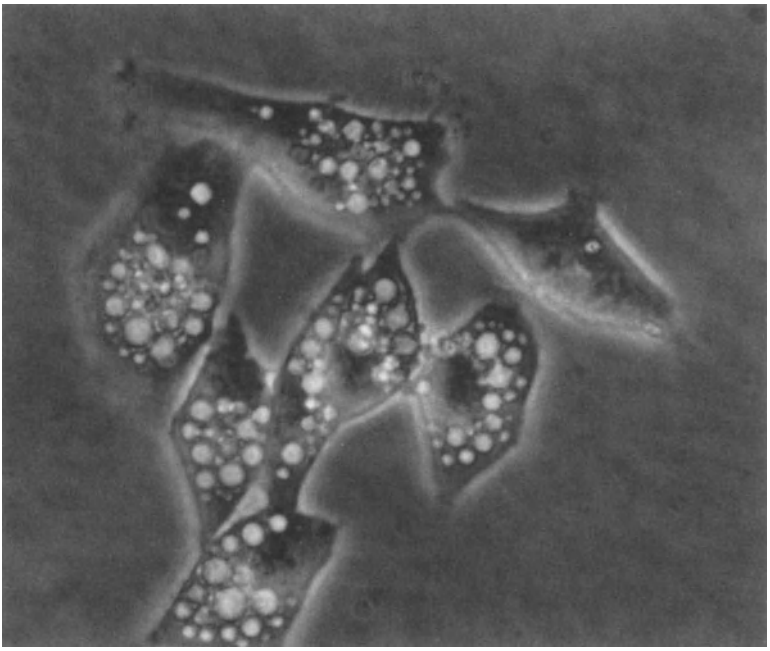

Fig. 1. Effect of vacuolating cytotoxin on HeLa cells. The cells were incubated for 5 h with a concentrated culture supernatant from *H. pylori* strain 185–44, producing a s1/m1 type of cytotoxin. Large cytotoxin-induced vacuoles are seen

Cover and Blaser reported the biochemical purification of the vacuolating cytotoxin from a *H. pylori*-culture supernatant, using a procedure including ammonium sulphate precipitation, hydrophobic interactive chromatography and size-exclusion- and anion-exchange chromatography. The cytotoxin, which could be purified more than 5000-fold, showed a molecular mass of approximately 90 kDa under denaturing conditions, whereas the native toxin was an aggregate with a molecular mass of approximately 1000 kDa (Cover and Blaser 1992). Antiserum raised against the purified, native cytotoxin and sera obtained from patients infected with cytotoxin-producing (tox^+) but not from cytotoxin-negative (tox^-) *H. pylori* strains neutralised the cytotoxic activity, indicating that the cytotoxin is produced in infected patients (Leunk et al. 1990; Cover et al. 1993b). Furthermore, cytotoxin production by *H. pylori* isolates in vitro correlates with cytotoxin production in vivo (Cover et al. 1993a). Culture supernatants of the toxinogenic *H. pylori* strain 60190 induced the formation of vacuoles in primary epithelial cells prepared from normal human mucosa (Harris et al. 1996; Smoot et al. 1996).

C. Gene Structure and Mechanism of Secretion

I. Cloning and Molecular Characterisation of *vacA* Encoding the Vacuolating Cytotoxin

The gene encoding the vacuolating cytotoxin has been cloned from independent cytotoxin-producing *H. pylori* isolates and is termed *vacA* (Cover et al. 1994; Phadnis et al. 1994; Schmitt and Haas 1994; Telford et al. 1994b). The cloning was facilitated by screening of a *H. pylori* gene library, which was performed with a gene probe deduced from the N-terminal amino acid sequence of the purified cytotoxin. Insertional mutagenesis of *vacA* using the mini-transposon Tn*Max5* or a kanamycin resistance cassette in the chromosome of a cytotoxin-producing *H. pylori* strain abrogated the production of cytotoxin activity (Cover et al. 1994; Schmitt and Haas 1994), thus confirming that *vacA* is essential for the vacuolating activity. DNA sequencing of three *vacA* genes surprisingly revealed an open reading frame encoding a 139-kDa to 140-kDa protein; this was in contrast to the mature extracellular VacA protein, which was in the range of 87–95 kDa (in the following, we refer to the 87-kDa to 95-kDa mature cytotoxin subunit as the 95-kDa protein). Thus, VacA was produced as a precursor molecule (Fig. 2). Primer extension located the transcriptional start site of *vacA* 114 bp upstream from the AUG start codon. All sequenced *vacA* genes contained a putative –10 box, but no –35 region was present (Schmitt and Haas 1994; Forsyth et al. 1998). Such atypical promoter sequences are found for many *H. pylori* genes. Furthermore, the overall sequence of the deduced VacA precursor protein did not show striking primary sequence homology with any known bacterial toxin.

The VacA precursor consists of several functional domains, which are cleaved during maturation to obtain an active cytotoxin. A 33-amino-acid

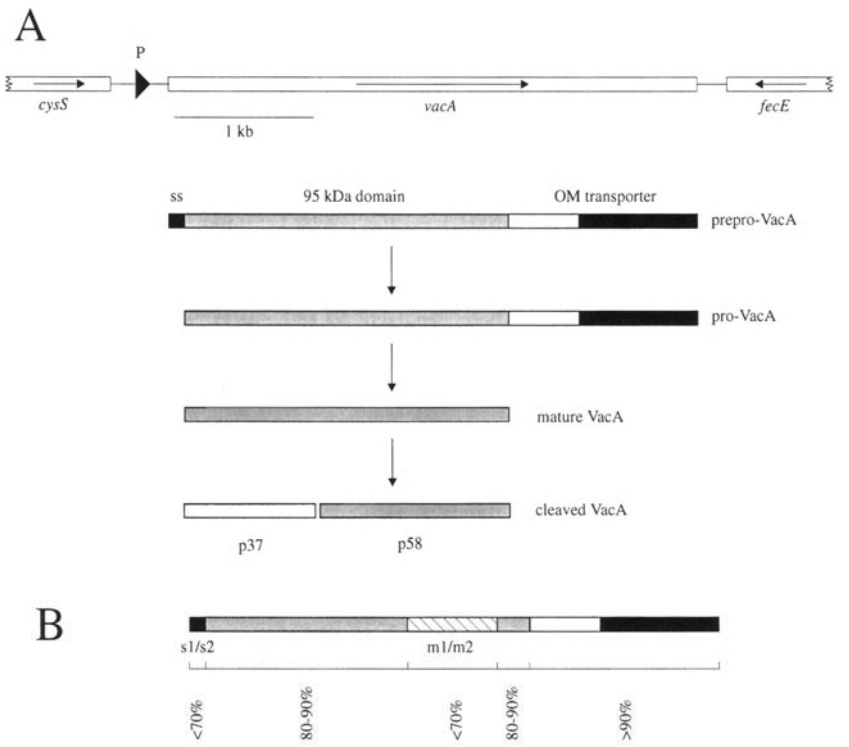

Fig. 2. Structural organisation of the *vacA* gene, processing of the VacA precursor protein and mosaic structure. **A** The *vacA* gene encodes a precursor protein of 140 kDa (prepro-VacA). The precursor consists of a cleavable signal sequence, the secreted 95-kDa protein and the cell-associated outer-membrane transporter. Further cleavage of the 95-kDa secreted protein might occur in the N-terminal p37 and C-terminal p58 fragments, which presumably corresponds to the A and B subunits of the toxin, respectively. **B** The degree of amino acid identity between s1/m1 and s2/m2 *vacA* alleles is indicated below. *s1/s2*, signal sequence type; *m1/m2*, mid-region type; *P*, *vacA* promoter, *cysS*, gene for cysteinyl transfer RNA synthetase; *fecE*, iron (III) dicitrate adensosine triphosphate-binding cassette transporter

leader sequence is removed through the cytoplasmic membrane by the *sec*-dependent translocation of the precursor (Fig. 2). The following 95-kDa domain constitutes the mature cytotoxin, which can be purified from the supernatant of *H. pylori* tox$^+$ strains. In certain *vacA* alleles, the 95-kDa subunit is further proteolytically processed into an approximate 37-kDa N-terminal and an approximate 58-kDa C-terminal fragment (Telford et al. 1994b). This latter cleavage occurs at a hydrophilic region between an alanine and a lysine within a short, almost perfect repeat of the sequence AKNDKXES. The repeat sequence is not present in all *vacA* sequences, and the cleavage of the 95-kDa protein is not observed in all VacA molecules studied (Schmitt and Haas 1994). As a third domain, the VacA precursor carries an extension of about

50kDa not found in the mature, secreted and active 95-kDa protein (Fig. 2). This C-terminal domain of the VacA precursor, which we refer to as the outer-membrane transporter (OM transporter), is involved in the secretion process of VacA through the OM of the gram-negative bacterium.

II. Autotransporter Organisation of the VacA Precursor

To study the role of the OM transporter of the VacA precursor protein, various Tn*Max5* transposon insertions into the OM-transporter sequence were generated. The resulting mutants failed to secrete any detectable 95-kDa protein, indicating that the C-terminal domain is essential for secretion of cytotoxin (Schmitt and Haas 1994). A secondary-structure prediction of the OM transporter revealed that this region contains a high proportion of amphipathic β-pleated sheets and terminates with a phenylalanine (Struyvé et al. 1991), indicating that this part of the VacA precursor is inserted into the OM of *H. pylori*. Furthermore, antisera raised against peptides from the C-terminal region of the precursor protein recognised an approximate 33-kDa fragment in the membrane fraction but not in the supernatant of the bacteria (Telford et al. 1994b).

Taken together, the structural organisation and the processing of VacA is characteristic of a family of surface-exposed or secreted proteins found in a number of diverse gram-negative pathogens (Jose et al. 1995). These proteins are characterised by the autotransporter (AT) mechanism, originally described for IgA proteases of pathogenic *Neisseria* species (Pohlner et al. 1987). AT proteins retain the ability to be translocated through both bacterial membranes without the help of separately encoded factors (Jose et al. 1995). The C-terminal portion of VacA shows only little primary-sequence homology with the IgAβ core, the minimal structural unit required for translocation of the neisserial IgA protease, but it also folds into a so-called β-barrel in the OM (Klauser et al. 1993; Schmitt and Haas 1994). Thus, it seems likely that both proteins are secreted by similar mechanisms.

A fundamental difference seems to exist, however, between the OM of *H. pylori* and other gram-negative bacteria, like *Escherichia coli* or *Neisseria gonorrhoeae*. In contrast to the AT proteins of many bacteria, the VacA precursor cannot be functionally produced in *E. coli*. The insertion of the C-terminal domain into the OM seems to be lethal for *E. coli*, indicating that there are fundamental differences between the *E. coli* and *H. pylori* OMs. One known difference is that *H. pylori* OM contains cholesteryl glucosides (Hirai et al. 1995), which is very rare in the bacterial world.

III. Mosaic Gene Structure of *vacA* Alleles in the *H. pylori* Population

Approximately 50% of *H. pylori* isolates produce a vacuolating cytotoxin activity in vitro, as measured in a cell-culture assay based on the HeLa cell

line or other cell lines. Southern hybridisation and polymerase-chain-reaction amplification of *vacA* alleles from tox$^+$ and tox$^-$ strains indicated that all *H. pylori* strains tested carried *vacA* gene sequences (Cover et al. 1994; Phadnis et al. 1994; Schmitt and Haas 1994). An immunoreactive 95-kDa cytotoxin was present in the culture supernatants of some tox$^-$ strains (Atherton et al. 1995), but other strains failed to show a cross-reactive band in the immunoblot (Schmitt and Haas, 1994; Xiang et al. 1995). The cloning and sequencing of a complete *vacA* gene from a tox$^-$ isolate showed, however, that a 142-kDa VacA protein is encoded by the corresponding *vacA* gene. The alignment between tox$^+$ and tox$^-$ VacA amino acid sequences revealed major divergence in the signal sequence and the mid-region of *vacA*, whereas the C-terminal, membrane-associated portion and the N-terminal region were very conserved between the two sequences (Atherton et al. 1995). Two major types of signal sequences and mid-regions were detected, designated as s1 and s2, and m1 and m2, respectively (Fig. 2). The s1 region was further divided into s1a and s1b. The mid-region spans approximately 300 amino acids, and m1 and m2 sequences have only 55% amino acid identity in this region. The m2 mid-region contains a 20-amino-acid deletion and a 23-amino-acid insertion compared with the tox$^+$ VacA sequence. The deletion covers the sequence duplication containing the exposed protease-cleavage site, which splits the 95-kDa secreted cytotoxin molecule into 37-kDa and 58-kDa fragments. The 23-amino-acid insertion carries a potential adenosine triphosphate/guanosine triphosphate (ATP/GTP)-binding motif (Walker et al. 1982), which is not present in the m1 sequence (Atherton et al. 1995).

IV. Consequences of the *vacA* Mosaic Gene Structure

The m2 allele located in the middle of the p58 fragment has generally been associated with an inactive cytotoxin associated with a tox$^-$ strain. Surprisingly, Pagliaccia et al. reported recently that the *vacA*/m2 allele of strain 95–54 has a cell-type-specific vacuolating activity for primary gastric cells and other cell lines, such as RK-13, a rabbit kidney epithelial cell line (Pagliaccia et al. 1998). The 58-kDa fragment of VacA is believed to be involved in binding of the toxin to a specific receptor on epithelial cells. Thus, it seems that the active domain of VacA can be delivered by different receptor-binding domains into distinct types of cells. Assuming that all *vacA*/m2 genotypes are associated with cytotoxic activity towards gastric epithelium, the former distinction of *H. pylori* strains in tox$^+$ and tox$^-$, based on vacuolation assays using HeLa cells, would have to be reconsidered. Only the minority of isolates producing no VacA protein should be regarded as true tox$^-$ strains.

V. Presence of *vacA* Homologues in the *H. pylori* Genome

Recently, the complete genome sequence for *H. pylori* 26695 was published (Tomb et al. 1997). In addition to the *vacA* gene (Hp0887), three *vacA*-

homologous genes encoding very large putative proteins with 1974 (Hp0619), 2529 (Hp0922) and 2932 amino acids (Hp0289) were identified. The sequence similarity to VacA is between 26% and 31% and is mainly concentrated on the C-terminal portion of the VacA precursor. A vacuolating activity was not attributed to these putative proteins, indicating that they might have a similar AT mechanism of export, rather than a functional homology like the vacuolating-cytotoxin activity.

D. Regulation of *vacA* Gene Expression

Only 50% of clinical isolates of *H. pylori* produce a cytotoxic activity in a HeLa cell vacuolation assay. As mentioned above, the *vacA*/m2 genotype is inactive in the HeLa vacuolation assay but gives active cell vacuolation in primary gastric cells and other cell lines. Forsyth and co-workers (1998) have shown that the level of transcription of the *vacA* gene from eight s1/m1 genotypes was significantly higher than in nine s2/m2 *H. pylori* strains. A *vacA*::*xylE*-reporter gene fusion coupled with the promoter of a tox^+ strain resulted in an approximately 30-fold-higher XylE activity as compared with the same construct behind the *vacA*-promoter of a tox^- strain. The different levels of VacA found extracellularly could, however, not solely be explained by different promoter strengths. The stability of *vacA* mRNA, depending on the signal-sequence type, the mid-region and the efficiency of secretion by the signal-sequence type, might play further crucial roles for the amount of active cytotoxin secreted extracellularly (Forsyth et al. 1998).

Of those strains which do not show vacuolating activity upon HeLa cells, some possibly produce a cytotoxin with different receptor specificity, and some strains do not produce a detectable VacA at all. With the exception of two *H. pylori* isolates (Phadnis et al. 1994), all strains analysed so far contain a *vacA* gene. Xiang et al (1995) analysed 43 *H. pylori* isolates for the presence of VacA protein (in a Western blot) and vacuolating activity. Fifteen strains did not produce detectable VacA protein. This fact suggests that other mechanisms of expression of *vacA* might exist. We have determined the complete sequence of a *vacA* gene that does not produce a VacA protein. That the 95-kDa protein was missing could be easily explained by a deletion of a single nucleotide corresponding to the beginning of the 37-kDa fragment in the *vacA* gene sequence, resulting in a premature termination of translation (Fischer and Haas, unpublished). Whether this mutation occurred in the *H. pylori* isolate in the patient or after cultivation and whether this is a general mechanism is not known yet.

E. Extracellular Structure and Activation of the Vacuolating Cytotoxin

I. Processing and Quaternary Structure

After secretion and processing to the mature 95-kDa cytotoxin, VacA might be processed further into two fragments of about 37 kDa and 58 kDa. This cleavage was especially observed upon prolonged storage (Telford et al. 1994b), but it might be relevant in vivo as well. The processing site has been determined, in strain NCTC11638, to be in a hydrophilic loop region within a short, almost perfect repeat of the sequence AKNDKXES. This sequence duplication is absent from all other *vacA* genes sequenced so far, but a similar proteolytic cleavage can be observed in the corresponding strains as well (Garner and Cover 1996). The two VacA fragments are linked non-covalently and can be co-purified. Even in strain NCTC11638, cleavage does not seem to be necessary for vacuolating activity, since deletion of the hydrophilic loop had no influence on toxin activity (Burroni et al. 1998).

These structural data suggest similarities with the AB family of dichain toxins. The 37-kDa fragment represents the enzymatically active A portion, and the 58-kDa fragment represents the receptor-binding B portion (Lupetti et al. 1996). However, other features of AB toxins, such as the covalent linkage of A and B portions by a disulphide bridge, are not found for VacA.

In solution, VacA forms oligomeric complexes with a molecular weight of at least 600 kDa (Manetti et al. 1995) but possibly more than 1 MDa (Cover and Blaser 1992; Cover et al. 1997). The surface structure of these complexes has been determined in two studies by quick-freeze, deep-etch electron microscopy, revealing a 30-nm-diameter flower-like structure with six- or sevenfold radial symmetry, in which a central ring is surrounded by petal-like extrusions (Fig. 3; Lupetti et al. 1996; Cover et al. 1997). The VacA molecule from strain NCTC11638 produced mainly structures with a sevenfold symmetry, which were interpreted as being VacA heptamers, but also produced structures with a sixfold symmetry interpreted as hexamers (Lupetti et al. 1996; Lanzavecchia et al. 1998). Another study used VacA from strain 60190, yielding almost exclusively hexagonal structures, which were interpreted as dodecamers by determining their thickness and by using glycerol density-gradient centrifugation (Cover et al. 1997). Whether the difference between these VacA structures is due to the presence (NCTC11638) or absence (60190) of the hydrophilic loop structure is unclear, but it has been reported that the VacA protein from strain NCTC11638 also forms predominantly hexagonal structures when its hydrophilic loop is deleted (Burroni et al. 1998).

II. Activation by Acid

A remarkable feature of VacA is its ability to become resistant against acid and pepsin. This obviously makes sense for a cytotoxin produced and secreted

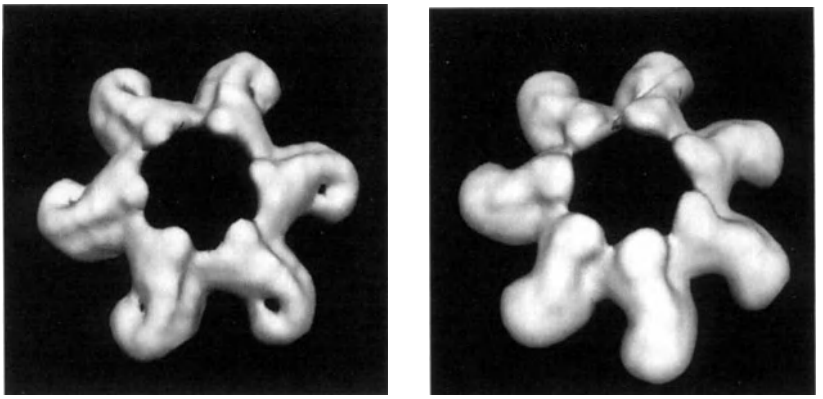

Fig. 3. Three-dimensional reconstruction of a hexamer and a heptamer VacA molecule. Quick-freeze deep-etched preparations were reconstructed from a metal mold using a tomographic technique, as described in LANZAVECCHIA et al. (1998). Courtesy of S. LANZAVECCHIA and J. TELFORD

in the gastric-mucus environment, where low pH and proteases are ubiquitous. As shown by DE BERNARD et al. (1995), an activation by acid is even necessary to obtain fully active cytotoxin. The acid activation, which is induced in vitro by a 10-s exposure of the molecule to a pH of 2 at 37°C, is accompanied by a structural reorganisation of VacA, as suggested by altered circular-dichroism and fluorescence spectra. These conformational changes are not reversed upon reneutralisation, but they seem to proceed to a third conformational state of the molecule (DE BERNARD et al. 1995). When VacA adsorbed to mica flakes for quick-freeze, deep-etch microscopy is exposed to a pH of 3.0, it changes its appearance to a loose, flower-like array of up to 12 petals (COVER et al. 1997). Pre-incubation at pH 3.0 results in a more or less complete dissociation of the oligomers. These changes in quaternary structure, however, can be reversed upon re-exposure to neutral solutions. A major determinant of conformational change upon acid activation might be exposure of hydrophobic stretches on the protein surface, which may be needed for penetration of lipid bilayers (MOLINARI et al. 1998a).

F. Effects of VacA on Eucaryotic Cells

I. Binding to Target Cells and Mechanism of Uptake

For cell intoxication to occur, bacterial toxins have to bind fairly specifically to cell-surface receptors. Subsequently, most AB-type toxins are internalised by endocytosis before translocation of the enzymatically active A compound to the cell cytosol is achieved. The interaction of VacA cytotoxin with lipid membranes and with the surfaces of eukaryotic cells has been demonstrated.

Recombinant VacA fragments corresponding to the putative p37 and p58 subunits produced in *E. coli* were able to induce a reversible aggregation of small unilamellar lipid vesicles at neutral pH and induced their fusion at a pH of 4.5 (Moll et al. 1995). Moreover, p58 is able to cause potassium efflux from liposomes at a pH of 4.5 but not at a pH of 7.4, suggesting that the p58 subunit is able to build up a transmembrane channel without a specific cellular receptor. As shown by photolabelling experiments, acid-activated VacA penetrates more deeply into lipid bilayers, which might be a prerequisite for channel formation and toxin translocation (Molinari et al. 1998a).

Cytotoxin binding to the surface of HeLa cells could be demonstrated by immunofluorescence (Garner and Cover 1996). Binding can be saturated, with a dissociation constant in the nanomolar range, suggesting the existence of specific cellular receptors (Massari et al. 1998). A cellular receptor for VacA has not yet been characterised, but a biotinylated surface protein of 140 kDa has been precipitated from lysates of VacA-susceptible cells (like human gastric-cancer cell lines AZ-521 and AGS), which might act as receptors (Yahiro et al. 1997). Vacuolation can be inhibited by pre-treatment of HeLa cells with an antibody reacting against epidermal-growth-factor receptor, suggesting an involvement of this receptor in VacA binding and/or uptake (Seto et al. 1998).

The VacA domain responsible for receptor binding has not been clearly defined. Recombinant VacA fragments corresponding to the putative 37-kDa and 58-kDa subunits were both found to bind to the surface of HeLa cells (Garner and Cover 1996). Binding of native VacA could be inhibited by pre-incubation of the culture supernatant with an anti-VacA antiserum. This was also possible using an antiserum against the recombinant 58-kDa fragment but not with an antiserum against the recombinant 37-kDa fragment, suggesting that binding is primarily achieved by regions in the p58 domain. However, since the native conformation of VacA may be necessary for the induction of neutralising antibodies (Manetti et al. 1995), this conclusion is uncertain.

After 4 h of incubation at 37°C, the cytotoxin can be identified inside HeLa or AGS cells, where it seems to be located in a perinuclear region, but not inside the vacuoles (Garner and Cover 1996). Various compounds known to inhibit cellular trafficking, such as brefeldin A or cytochalasin D, or to inhibit cytotoxin vacuolation, such as bafilomycin A1 or monensin, did not influence VacA uptake. Whether endocytosis is necessary prior to translocation to the cell cytosol is not known. Exposure to an acidic intracellular compartment does not seem to be necessary, since the presence of ammonia increases rather than inhibits cell vacuolation (Cover et al. 1991). VacA does induce vacuole formation from the cell cytosol, as demonstrated by microinjection of purified VacA and by transfection of epithelial cells with a plasmid encoding the complete 95-kDa domain of VacA (De Bernard et al. 1997). However, neither the p37 fragment nor the p58 fragment nor both expressed together were able to induce vacuolating activity in transfected cells. Thus, it cannot be decided yet

whether or not p37 represents the enzymatically active A subunit and p58 the receptor-binding and translocating B subunit, as suggested.

II. Vacuole Formation

The formation of vacuoles is the most obvious effect of VacA on epithelial cells, although this might not be its sole in vivo function. Vacuolation is a rather slow process, being visible at about 90 min after VacA treatment (Cover et al. 1992a). Vacuolated cells are able to exclude trypan blue for a considerable time, but prolonged exposure eventually leads to cell death. The size of the vacuoles reaches up to several micrometers, suggesting a fusion of smaller compartments and continuous addition of membranes.

Vacuoles are readily stainable by lysosomotropic weak bases, such as neutral red or acridine orange, which indicates an acidic vacuolar lumen (Cover et al. 1991; Catrenich and Chestnut 1992). Staining with neutral red has been widely used as a method for densitometric quantification of vacuolation (Cover et al. 1991). The vacuolating effect can be inhibited and even reversed by bafilomycin A1, suggesting that the activity of a vacuolar ATPase is essential (Cover et al. 1993c; Papini et al. 1993) and underlining the necessity of intracellular pH gradients. The ionophore monensin also inhibits vacuolation, whereas ouabain and digoxin, inhibitors of the Na^+–K^+ ATPase, and nicotine potentiate the vacuolating activity (Cover et al. 1992b, 1993c).

In early ultrastructural studies, vacuoles were suggested to originate from an autophagosomal compartment (Catrenich and Chestnut, 1992; Cover et al. 1992a), but vacuole formation could not be inhibited by the autophagy inhibitor 3-methyladenine (Papini et al. 1994). In contrast, vacuolation is inhibited by the microtubule-depolymerising agents nocodazole and colchicine. Vacuolar membranes are enriched in the small GTP-binding protein rab7 (Papini et al. 1994). Rab7 is involved in transport from early endosomes to late endosomes and from late endosomes to lysosomes, suggesting a late-endosomal origin of VacA-induced vacuoles (Fig. 4A). In accordance with this observation, vacuoles contain fluid-phase markers, although, at early time points, they seem to be endocytically hypoactive, excluding the pinocytosis marker Lucifer yellow (Catrenich and Chestnut, 1992; Cover et al. 1992a).

Rab7 is not merely a marker for the vacuoles but is also necessary for vacuole formation. Wild-type cells or cells expressing constitutively active rab7, but not dominant-negative rab7 mutants can be vacuolated by VacA (Papini et al. 1997). Rab5 also seems to be involved to a certain extent, as cells expressing constitutively active rab5 are protected from vacuolation. Cells constitutively expressing rab9, however, are vacuolated just as much as wild-type cells (Papini et al. 1997). These observations do not allow discrimination between effects on cytotoxin uptake and effects on cytosolic activity. However, with the demonstration that transfected cells expressing cytosolic VacA develop vacuoles (De Bernard et al. 1997), it could be shown that vacuolation is inhibited in dominant-negative rab7 mutants independent of toxin

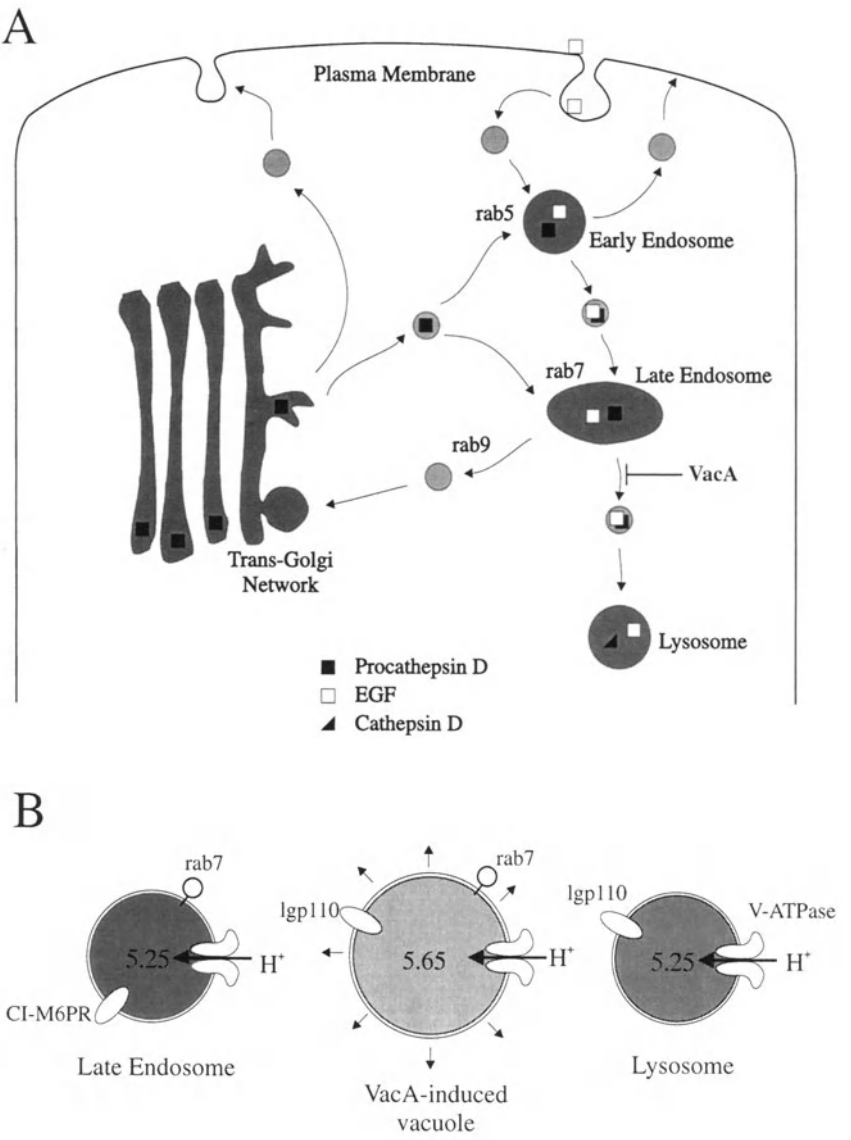

Fig. 4. Intracellular vesicle trafficking and the action of VacA. **A** Schematic representation of some pathways of intracellular membrane trafficking. Transport vesicles linking the various compartments are shown as *small circles*. The locations of several rab proteins involved in vesicle targeting are indicated. VacA seems to exert a block at some stage of the late-endosome-to-lysosome transport, which causes improper transport and processing of the lysosomal protease procathepsin D (*black squares*) and which blocks transport and lysosomal degradation of epidermal growth factor (*EGF*; *white squares*; Satin et al. 1997). **B** VacA-induced vacuoles are intermediate compartments between late endosomes and lysosomes, containing some late-endosomal (rab7) and some lysosomal (lgp110) markers and exhibiting a slightly increased luminal pH (Molinari et al. 1997). *CI-M6PR*, cation-insensitive mannose-6-phosphate receptor; *lgp110*, lysosomal membrane glycoprotein; *V-ATPase*, vacuolar adenosine triphosphatase

uptake. Apart from rab7, VacA-induced vacuoles contain the lysosomal marker lgp110, but not the late-endosomal marker cation-insensitive mannose-6-phosphate receptor (Molinari et al. 1997), suggesting that they are intermediate compartments between late endosomes and lysosomes (Fig. 4B).

The function of the excessive vacuolation exerted by VacA is not known. Upon prolonged exposure to VacA, the gastric epithelium may be eroded, and necrotic cells might release nutritional compounds beneficial to the bacterium, but there are no hints that VacA-producing strains have an advantage over VacA-negative strains regarding colonisation, although type I strains generally reach a higher density (Atherton et al. 1996). In fact, in the gnotobiotic piglet model, expression of the vacuolating activity did not have any effect on colonisation (Eaton et al. 1997). Another possibility might be a subtle deviation of specific cellular functions, leading to a more favourable environment.

III. Other Effects of VacA

Many bacterial toxins have been assigned modulatory effects at concentrations far lower than what is necessary for their (primary) toxic activity. Some have been shown to influence the production and homeostasis of various cytokines, which has led to the notion that some bacterial exotoxins should be regarded as potential modulins, just like endotoxin or other bacterial-surface components.

At sublethal concentrations, VacA has an influence on cellular processes. The transport and proper processing of the lysosomal protease cathepsin D is precluded in VacA-treated cells, as is uptake and degradation of epidermal growth factor (EGF; Satin et al. 1997; Fig. 4A). Cell-surface recycling of transferrin and endocytosis and retrograde transport of diphtheria toxin and ricin was not altered. All these effects can be explained by supposing a block in late-endosomal-to-lysosomal trafficking, which would lead, in the end, to accumulation of the observed hybrid vesicles but which would, at an earlier stage, modify many cellular trafficking pathways. One possible function of such an intervention with vesicle trafficking was suggested recently when it was shown that VacA is able to inhibit the invariant-chain (Ii)-dependent antigen-presentation pathway by B cells but not the antigen presentation of recycling major histocompatibility complex class-II molecules (Molinari et al. 1998b). Whether this might result in differential stimulation of T helper cell subpopulations remains to be determined.

VacA may not only damage gastric mucosal cells, it may also impair gastric-mucosal healing, thereby contributing to the development of ulcers. Indeed, VacA was shown to have an inhibitory effect on MKN28 cell proliferation (Ricci et al. 1996). An important factor in repair processes in the gastric mucosa is EGF. Kato-III cells respond to EGF by enhanced expression of the EGF receptor, increased receptor phosphorylation, activation of the extracellular-signal-regulated-kinase signalling cascade and an increased level of c-fos. All these effects can be inhibited by culture supernatants of the VacA-

producing strain 60190 but not by an isogenic mutant (Pai et al. 1998). However, *Helicobacter pylori*-culture supernatants are able to induce upregulation of the EGF-related peptides amphiregulin and the heparin-binding EGF-like growth factor in MKN28 cells independently of VacA (Romano et al. 1998). This effect, however, is not able to overcome the proliferation inhibition. An explanation for the impairment of EGF function may be the recent finding that VacA seems to interact with the EGF receptor (Seto et al. 1998).

G. Clinical Relevance of the Vacuolating Cytotoxin

In 1989, Figura and co-workers compared the cytotoxin production of *H. pylori* isolates from gastritis patients with those from ulcer patients in a small group of 77 patients. They found cytotoxin production in 66.6% of strains isolated from ulcer patients and 30.1% in strains from gastritis patients. This significant difference prompted the authors to suggest that the cytotoxin could be involved in the development of peptic ulcers (Figura et al. 1989). Later, Phadnis and co-workers noted that, in a small group of 27 *H. pylori* isolates, all patients infected with cytotoxin-producing strains had gastritis, and 78% had polymorphonuclear infiltration of the gastric mucosa. In the cytotoxin-negative group, however, only 33% had polymorphonuclear infiltration of the gastric mucosa, and 22% were found to have normal mucosa (Phadnis et al. 1994). Furthermore, *cagA*$^+$/*vacA*/s1 strains were found to colonise antral gastric mucosa at a fourfold-higher density compared with *cagA*$^-$/*vacA*/s2 strains (Atherton et al. 1996). The same authors suggested a significant correlation between the presence of the *vacA*/s1 genotype and the presence of peptic ulcers (Atherton et al. 1995). A similar correlation was found recently between the infection with *vacA*/s1 strains and gastric cancer in Germany (Miehlke et al. 1998).

In contrast to these results, other investigators did not find an association between different *vacA* genotypes and gastric or duodenal ulcer disease (Kodama et al. 1996; Go et al. 1998). The data obtained from different animal models were also not able to solve this question. Thus, gnotobiotic piglets could be successfully colonised with either a toxigenic *H. pylori* or a non-toxigenic isogenic mutant, but no difference in gastroduodenal disease was observed between the groups (Eaton et al. 1997). Telford et al. (1994b) and Ghiara et al. (1995) reported, however, that extracts from cytotoxic type-I strains, or purified cytotoxin, induced various epithelial lesions in the stomach mucosa of mice, like vacuolation, erosions and ulcerations. Non-cytotoxic strains induced mucin depletion but no other significant pathology. In this study, a considerable amount of extract or cytotoxin was given to the animals.

In conclusion, a clear functional association between *vacA* expression and clinical outcome of any type of gastroduodenal disease cannot be drawn yet. More studies are necessary, and larger patient groups from different geo-

graphical areas should be compared to obtain statistically significant data before clinical relevance can be determined.

H. VacA as a Vaccine Candidate

H. pylori can be adapted to colonise the stomachs of mice. Infections with type-I strains result in a gastric pathology similar to that seen in human disease (Marchetti et al. 1995), comprising a loss of gastric-gland architecture, erosions and ulcerations of the gastric epithelium and infiltration of the lamina propria with inflammatory cells, but with a lower degree of polymorphonuclear granulocytes. Using this mouse model, it was shown that immunisation with purified VacA together with *E. coli* heat-labile enterotoxin as an adjuvant protected mice from infection with a type-I strain (Marchetti et al. 1995, 1998).

Development of an immune response against VacA seems to be dependent upon native conformation (Manetti et al. 1995). Antisera developed against recombinant VacA are unable to neutralise the vacuolating activity and do not recognize native VacA in an enzyme-linked immunosorbent assay. Native VacA, however, can be detoxified by mild formaldehyde treatment (Manetti et al. 1997). Formylated VacA is still able to bind to target cells but has no vacuolating activity. It was successfully used for protection experiments, although the induction of neutralising antibodies was somewhat impaired in comparison to the case when untreated VacA is used.

Recombinant VacA in combination with LTK63, a non-toxic mutant of heat-labile enterotoxin, was used successfully for therapeutic vaccination (Ghiara et al. 1997) and for mucosal immunisation in the mouse model (Marchetti et al. 1998). LTK63 preferentially provokes a Th0 and Th2 response, which might be necessary for successful eradication, in contrast to the usually predominant Th1-type response observed in *H. pylori* infections. Antigen-specific protection was observed, but for type-I strains only.

I. Concluding Remarks

Ten years have passed since the first description of the *H. pylori* cytotoxin. Meanwhile, the structural and biological data suggest that the cytotoxin represents a new member of the bacterial AB type of toxins. The cytotoxin-encoding gene *vacA* has two remarkable features: its autotransporter structure and its allelic variation or mosaicism. Thus, the amino acid sequence variations in the signal sequence (s1/s2) and mid-region (m1/m2) apparently determine the efficiency of secretion of VacA and the receptor specificity of the exported mature cytotoxin. The assembly into a supramolecular structure seems to be a prerequisite to obtain cytotoxic activity. The binding and internalisation step is certainly not understood yet. Even less is known about the mechanism of vacuole formation and other effects of VacA. The specific target

molecule of the cytotoxin (in the eucaryotic cell) leading to the vacuolating phenotype is not known. New techniques have been developed recently, like the expression of active VacA domains inside the eucaryotic cell; these techniques make it possible to use (genetically) modified versions of VacA for the identification of the target molecule and the (enzymatic) activity associated with this extraordinary cytotoxin.

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CHAPTER 22

Staphylococcal α Toxin

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A. Occurrence and Biological Significance

Staphylococcal α toxin is a major pathogenicity determinant of *Staphylococcus aureus* (Bhakdi and Tranum-Jensen 1991). It is not produced by coagulase-negative staphylococci, and this is probably one reason for their reduced virulence compared with *S. aureus*. The toxin is secreted as a 293-residue water-soluble monomer of molecular weight 33000 Da (Gray and Kehoe 1984). Gene expression is subject to complex regulation (Recsei et al. 1986). Antibodies against α toxin become detectable in sera of all young adults, indicating that toxin production occurs in humans even in the absence of clinical disease. Similarly, antibodies can be found in sera of domestic animals, reflecting the fact that *S. aureus* is a most versatile pathogen that infects a broad spectrum of hosts. *S. aureus* infections range from self-limiting skin afflictions to life-threatening systemic diseases, and *S. aureus* is a major causative agent of hospital infections. Inasmuch as the pathogenesis of staphylococcal disease is multifactorial, α toxin probably assumes a dominant role in most cases. In animal models, purified toxin has been shown to be hemolytic, dermonecrotic, and lethal (McCartney and Arbuthnott 1978). The biological importance of toxin production has formally been shown by two approaches. First, the use of genetically deficient bacterial-mutant strains has proven α toxin to be a major cause of tissue destruction and abscess formation (Jonsson et al. 1985; Patel et al. 1987; Bramley et al. 1989; Menzies and Kernodle 1994). Second, the application of mono- and polyclonal antibodies against α toxin has been found to protect against development of staphylococcal lesions. α Toxin is thus not only an archetype of a channel-forming bacterial toxin, it constitutes the best-studied example of how a pore-forming toxin can contribute to microbial virulence (Bhakdi and Tranum-Jensen 1991; Bhakdi et al. 1996).

B. Purification and Properties of Monomeric Toxin

α Toxin can be recovered in culture supernatants of *S. aureus* strains. The classic high-level-producing strain Wood 46 is generally used for purification purposes. Naturally occurring toxin mutants have not been detected to date,

so the toxin gene is highly conserved. Somewhat surprisingly, α toxin represents a unique molecular entity, and no other molecule displaying extensive homology has yet been discovered. Restricted homologies do exist in the pore-forming domain, however; these have been noted in a family of bi-component, pore-forming leukocidins also produced by *S. aureus* (Supersac et al. 1993; Gouaux et al. 1997)

Under optimal culture conditions, α toxin comprises the major protein component in overnight bacterial supernatants. The bulk of the toxin is present in monomeric form. Purification is easy and can be achieved by single-step ion-exchange chromatography (Valeva et al. 1996) or by fast protein liquid chromatography (Lind et al. 1987). Toxin preparations can be transferred to ammonium acetate and then lyophilized. In this form, the protein is stable for years at –20°C and for weeks at room temperature. The A_{280} of a 1-mg/ml solution is approximately 2.0. The toxin is soluble in water or any aqueous buffer at a pH of 5–9. Native toxin migrates as a single polypeptide band of molecular weight 33000 Da in sodium dodecyl sulfate (SDS) polyacrylamide-gel electrophoresis. The pI is approximately 8.6 (Bhakdi and Tranum-Jensen 1991).

The three-dimensional structure of monomeric α toxin has not yet been solved. Analyses of secondary structure have shown that native toxin harbors an abundance of β-sheet structure (approximately 60%); approximately 10% is α-helical, and the rest consists of non-α/non-β elements (Tobkes et al. 1985). Rather unexpectedly, the membrane-inserting domain, which is located near the center of the polypeptide chain, is totally exposed to the hydrophilic environment (Valeva et al. 1995, 1996, 1997a, 1997b).

Monomeric α toxin is destroyed by proteases. Under controlled conditions, proteolytic cleavage first occurs in the pore-forming domain, generating a nicked molecule that still binds to form smaller pores in membranes of erythrocytes (Palmer et al. 1993b).

Native α toxin can be radioiodinated without loss of function (Hildebrand et al. 1991), and cysteine substitution mutants can be site-specifically labeled with fluorescent molecules, with biotin, or with radiolabeled fluorescein (Palmer et al. 1993a; Valeva et al. 1996, 1997a, 1997b). It is, therefore, easy to produce various probes for in vitro and in vivo studies.

C. Mechanism of Action

I. Binding

The toxin binds as a monomer in the absence of any molecular modification (Bhakdi and Tranum-Jensen 1991; Hildebrand et al. 1991). Binding studies have revealed that interaction with red blood cells can occur via two distinct mechanisms (Hildebrand et al. 1991). Highly susceptible cells, e.g. rabbit erythrocytes, express a relatively small number (approximately 2000 per cell) of high-affinity binding sites, which are responsible for the interaction of toxin

with membranes at low concentrations (2 nM; 60 ng/ml). Binding is optimal at ambient temperature (22–25°C). Both rabbit erythrocytes and human erythrocytes also display an unsaturable number of low-affinity binding sites, which account for the hemolytic properties of α toxin on less susceptible red blood cells (human erythrocytes). Neither the low- nor the high-affinity binding sites have been molecularly identified. They are not destructible by the action of proteinase K, pronase, phospholipase A_2, phospholipase C, phospholipase D or sphingomyelinase.

High-affinity binding sites are believed to be present on highly susceptible cells (keratinocytes, endothelial cells, lymphocytes, and monocytes) and on platelets, although this contention has not been proven experimentally. Current work indicates that the mode of toxin binding to nucleated cells is rather complex, and different susceptibilities are not explained on the basis of two binding sites alone.

II. Oligomerization

Toxin oligomers form as membrane-bound monomers diffuse laterally and collide with each other in the bilayer. Oligomerization occurs at 0°C (Valeva et al. 1997b), so membrane fluidity is of negligible significance. Oligomerization also occurs spontaneously in solution (Bhakdi et al. 1981, 1996). We have postulated that the main effect of membrane binding is to uniformly orient toxin monomers so their contact surfaces are correctly positioned when collision occurs (Bhakdi and Tranum-Jensen 1991). Non-circularized structures probably representing oligomeric intermediate stages have been detected by atomic-force microscopy on liposomes (Czajkowsky et al. 1998), but they have eluded biochemical characterization, possibly because they dissociate in detergents. Two different oligomers that successively assemble to form pre-pores before membrane-insertion occurs have been clearly defined. The first is an oligomer that is stable in non-denaturing detergents, such as deoxycholate, but dissociates in SDS. This early pre-pore complex forms on erythrocytes at 4°C. Pre-pores with similar properties can be generated at ambient temperature through the use of a substitution mutant in which His-35 is replaced by arginine. This substitution causes oligomer assembly to be blocked at this pre-pore stage. Application of H35R α toxin to cells consequently leads to blockade of high-affinity binding sites and to the generation of SDS-labile, non-functional oligomers (Jursch et al. 1994).

The first pre-pore stage is succeeded by a second stage resulting from cooperative effects within the oligomer complexes. Hydrophobic protein–protein interactions gain dominance, and an SDS-stable oligomer whose pore-forming domain is still located outside the bilayer is generated (Walker et al. 1992; Valeva et al. 1997b). Such late pre-pore complexes can form on certain cells that display a natural resistance towards toxin attack, e.g. on human granulocytes. These cells then carry large numbers of SDS-stable oligomers without becoming permeabilized (Valeva et al. 1997a).

III. Pore Formation

In a final step, assembled oligomers insert their pore-forming sequences into the bilayer, and a channel is formed (Fig. 1). There is evidence that this step is governed not by cooperative effects but primarily through conformational changes occurring within each of the protomers (Valeva et al. 1997c).

The diameters of channels created by α toxin have been measured as approximately 1 nm in planar lipid bilayers (Menestrina 1986; Belmonte et al. 1987) and in cell membranes (Kasianowicz and Bezrukov 1995). However, it seems that pore diameters are slightly smaller when susceptible cells (keratinocytes) are exposed to low toxin concentrations, because the cells then leak K^+ without permitting influx of Ca^{2+} (Walev et al. 1993). At higher toxin concentrations, slightly larger pores appear to be created, and rapid Ca^{2+} influx is noted (Walev et al. 1993; Jonas et al. 1994). The cause of this small heterogeneity in pore size has remained enigmatic. Initially, cytoplasmic proteins do not leak out of toxin-damaged cells.

D. Structure of Oligomeric Pores

I. Structure of the Heptameric Pore Formed in Detergent Solution

The key finding that ultimately led to crystallographic studies of the α toxin oligomer was made in 1981, when it was found that oligomerization of α toxin could be induced simply through the addition of certain detergents, such as deoxycholate (Bhakdi et al., 1991). Until today, the reason for this phenomenon has not been clarified. Since the ultrastructural appearance of detergent-induced oligomers did not noticeably differ from those forming on target membranes, it was assumed that the structures were identical.

Crystallization of the detergent-induced oligomer was achieved a decade later (Gouaux et al. 1994), and its three-dimensional structure was solved at 1.9-Å resolution (Song et al. 1996). The oligomer measures 10 nm in height and 10 nm in diameter and has the shape of a mushroom, with a 1-nm pore running through its center (Fig. 2). Each oligomer is composed of seven α-toxin molecules. The heptamer has cap, rim, and stem domains. The last of these is a 14 strand anti-parallel β-barrel which forms the wall of the putative transmembrane pore. The cap domain represents approximately 70% of the total mass of the oligomer and resides outside the bilayer. The rim domain underlies the cap and is probably in close contact with the outer-membrane leaflet.

The putative membrane-spanning stem domain measures 52 Å in height and 26 Å in diameter. Therefore, each protomer contributes two anti-parallel β-strands formed from the amino acid sequence 118–140. The side chains of the even residues in this sequence should contact lipid, whereas the side chains of the uneven residues would be located in the pore lumen. Residue 129

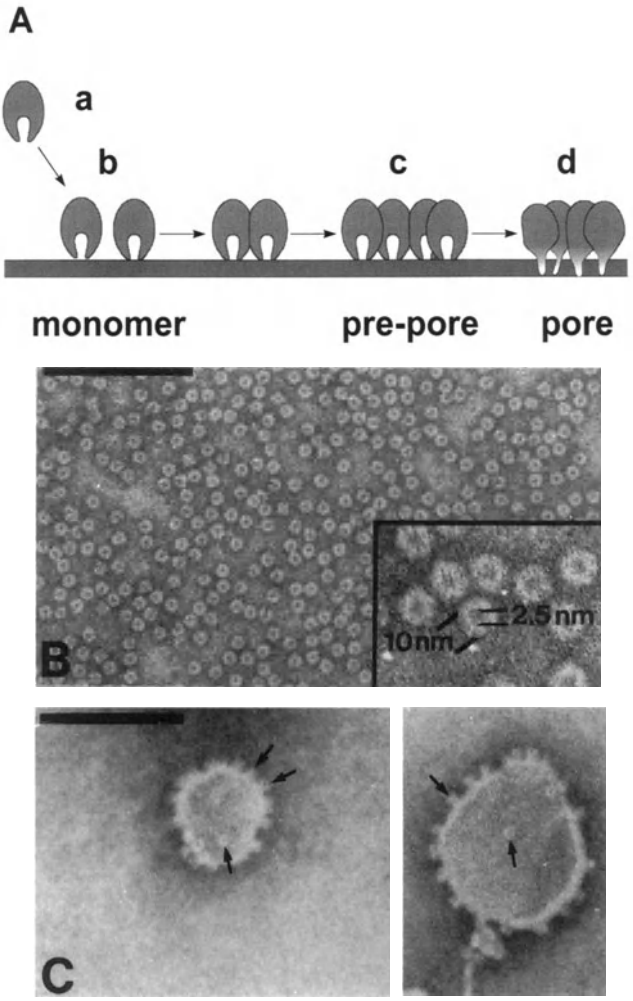

Fig. 1. **A** Assembly model for α toxin in lipid bilayers. Water-soluble native monomers (*a*) bind to and orient themselves on lipid bilayers (*b*). Membrane-bound monomers collide via lateral diffusion in the membrane plane to form pre-pore complexes (*c*). Oligomerization provides the diving force for insertion of the central molecular domain into the bilayer; a hydrophilic transmembrane pore traverses the center of the circularized, heptameric protein complex (*d*). **B** Isolated toxin heptamers in detergent solution. **C** Lecithin liposomes carrying reincorporated α-toxin heptamers. The heptamers are seen as stubs along the edge of the liposomal membrane and as rings over the membrane (*arrows*). *Bar* 100 nm (in **B** and **C**)

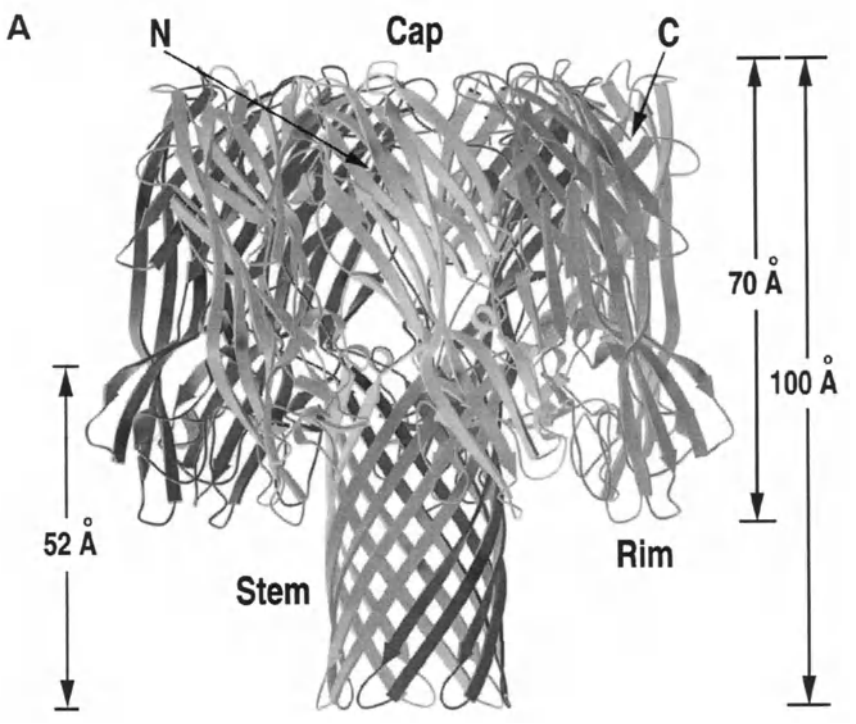

Fig. 2. Ribbon representations of the α-hemolysin heptamer. **A** View perpendicular to the sevenfold axis and approximately parallel to the putative membrane plane. The mushroom-shaped complex is approximately 100 Å tall and up to 100 Å in diameter, and the stem domain measures about 52 Å in height and 26 Å in diameter from C_α to C_α. Approximate locations of the cap, rim and stem domains are shown. Thr^{129} is located at the base of the stem domain. **B** View from the top of the structure and parallel to the sevenfold axis. The amino latch of one protomer makes extensive interactions with its clockwise-related immediate neighbor, and residues in each glycine-rich region wrap around the sevenfold axis approximately 180°. Protomer–protomer contacts consist almost exclusively of side-chain–side-chain interactions in the cap domain while, in the stem domain, main-chain–main-chain contacts predominate as the β strands form a continuous β sheet. Reproduced from Song et al. 1996 with permission

represents the turning point of each β sheet at the cytoplasmic face of the bilayer (Song et al. 1996).

II. Structure of the Membrane-Bound Oligomer

Given the clarity and beauty of the crystallographic studies, there appears to be little reason to question whether the structure is identical to that of pores forming on target membranes. In fairness, however, it must be conceded that this has not yet been rigorously proven. From earlier data, many groups had concluded that the oligomer forming on biological membranes was a hexamer

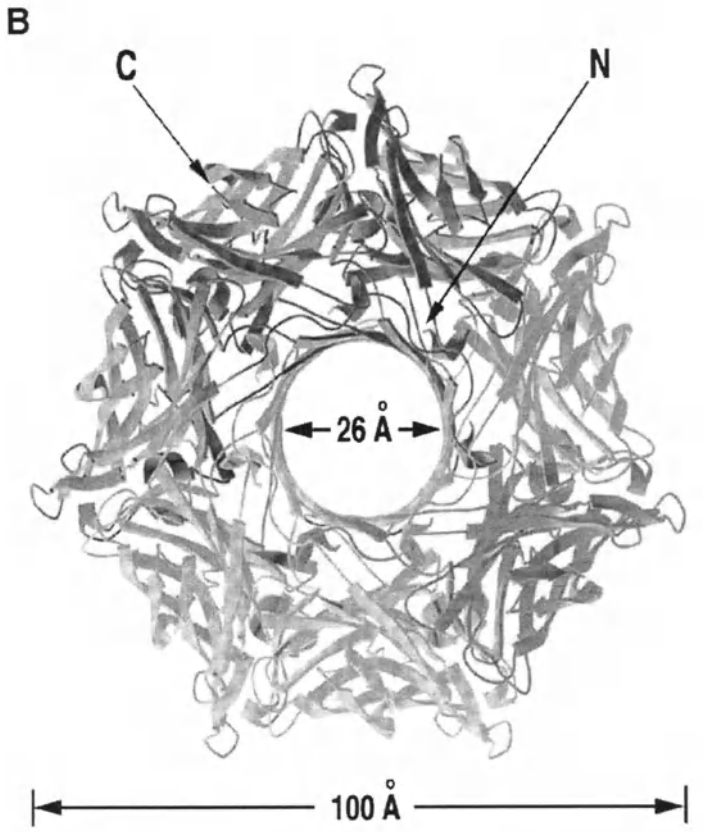

Fig. 2. *Continued*

(Freer et al. 1968; Bernheimer 1974; Füssle et al. 1981; Tobkes et al. 1985). Analyses of two-dimensional crystals on liposomes strengthened this contention (Ward and Leonard 1992) and, recently, atomic-force microscopy seemed to clearly reveal that this conclusion was correct (Czajkowsky et al. 1998). Thus, we may still be faced with an open question regarding the composition and molecular organization of α toxin in biological membranes.

Regardless of the true stoichiometry, however, spectroscopic studies strongly support the basic model emerging from the crystallographic analyses. In particular, there can be little doubt that the pore is indeed formed through insertion of a single amino acid sequence encompassing residues 118–140. The novel experimental strategy leading to in situ identification of this domain involved production of single cysteine substitution mutants that were derivatized with acrylodan, a sulfhydryl-specific, polarity-sensitive fluorescent dye. The emission spectrum of acrylodan depends on the polarity of its environment: the probe fluoresces green in water and blue in a hydrophobic envi-

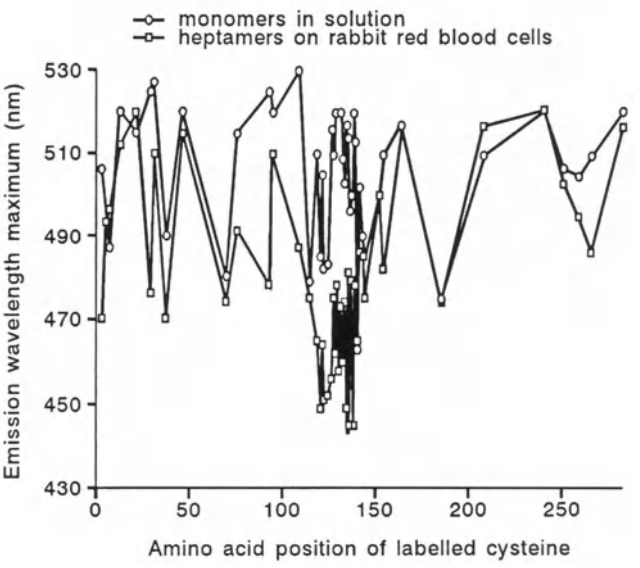

Fig. 3. Fluorescence-emission maxima of acrylodan-labeled α-toxin cysteine mutants in solution and after heptamer assembly on rabbit erythrocytes. An emission blue shift indicates movement of the fluorescence label to a more hydrophobic environment. Significant blue shifts were observed exclusively in the central region spanning residues 118–140

ronment. Analyses of 50 labeled toxin mutants revealed that, in emission spectra, only acrylodan attached to residues 118–140 exhibited a marked blue shift coincident with oligomer formation in lipid bilayers (Fig. 3). By employing mutant toxins exhibiting defects at defined stages of assembly, insertion of the pore-forming sequence could be shown to correlate with function. When susceptible cells were examined, periodicity in side-chain environmental polarity was detected; thus, every even residue in the sequence entered a nonpolar environment in a striking display of an amphipathic transmembrane β barrel. These data have resolved the molecular organization of the α-toxin channel in living cells, and they are in superb accord with the crystallographic data (Ward et al. 1994; Song et al. 1996; Valeva et al. 1997a, 1997b).

Should it be true that hexamers rather than heptamers assemble on biological membranes, the present crystallographic model would, of course, have to be modified somewhat. Necessarily, the central channel would also be slightly smaller. In fact, there have been many indications that membrane-bound α-toxin pores may display some small variation in size. When applied at low concentrations to susceptible cells, the pores appear to be slightly smaller than those generated at high toxin concentrations. The possibility

exists that pore heterogeneity reflects structural heterogeneity, with heptamers and hexamers forming alongside each other.

E. Biological Effects

I. Cytocidal Action

Cell death is the most obvious and inevitable consequence of pore formation if a lesion is not removed or repaired. Cell death can have immediate detrimental consequences. Tissue necrosis generates niches for bacterial survival and multiplication. The deaths of phagocytes and lymphocytes foster microbial persistence and invasion. Monocytes (Bhakdi et al. 1989) and lymphocytes (Jonas et al. 1994) are, indeed, prime targets for α-toxin attack. Endothelial cells are also highly susceptible (Suttorp et al. 1985; Seeger et al. 1990), and their deaths can lead to major perturbations in the microcirculation. Further, α toxin exerts potent cytocidal action on keratinocytes (Walev 1993), which coincides with the known relevance of *S. aureus* as a major cause of skin infections.

II. Secondary Cellular Reactions

Secondary reactions mounted by cells under attack by any pore-forming toxin are of prime importance, and they can explain the long-range effects of these toxins. The reactions provoked by α toxin may conveniently be grouped into two major categories.

1. Reactions Provoked by Transmembrane Flux of Monovalent Ions

Pores that allow passage of K^+ and Na^+ but that restrict passage of Ca^{2+} and larger molecules elicit unexpected effects. When α toxin is applied to activated T lymphocytes at low concentrations, such that Ca^{2+} flux is restricted, programmed cell death is triggered, and DNA fragmentation occurs after 2–4 h (Jonas et al. 1994). When applied at similarly low concentrations to monocytes, α toxin provokes the processing and transport of interleukin (IL)-1β (Bhakdi et al. 1989; Walev et al. 1995). The latter phenomenon is due to rapid cleavage of intracellular pro-IL-1β precursor by the membrane-bound IL-converting enzyme (ICE) caspase 1. Since ICE-related proteases are also involved in the apoptosis pathway, these two findings may be linked to one another. How monovalent ion flux is linked to caspase activity is not yet known. Recent data suggest that K^+ efflux enhances the autocatalytic activation of pro-caspase 1 (Cheneval et al. 1998). The intriguing concept emerging from these studies is that K^+ directly or indirectly controls the function of vitally important proteolytic enzymes in the cell. Both programmed cell death and IL-1β conversion were inhibited by high extracellular concentrations of K^+ in

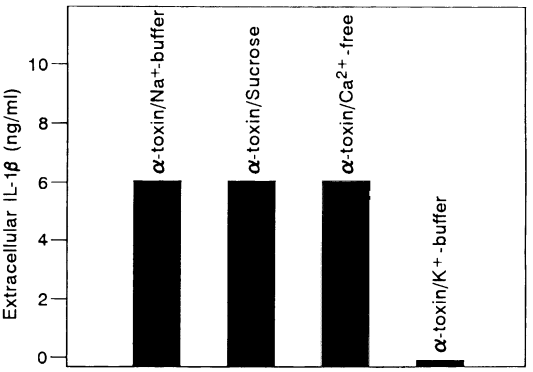

Fig. 4. Extracellular K^+ inhibits interleukin (IL)-1β maturation in α-toxin-permeabilized monocytes. Cells were stimulated with lipopolysaccharide for 90 min and then transferred either to Na^+ buffer, 20 mM 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid/260 mM sucrose, Na^+ buffer without Ca^{2+} with 2 mM ethylene diamine tetraacetic acid or K^+ buffer, and permeabilized with 1 μg/ml α toxin. After 60 min, the supernatants were assayed for extracellular IL-1β. Neither Na^+ nor Ca^{2+} influx was required for triggering IL-1β maturation. No maturation occurred in the presence of KCl in the medium

α-toxin-permeabilized cells (Fig. 4), and recent work emphasizes that K^+ efflux is an early event occurring in apoptotic cells (Bortner et al. 1997; Hughes et al. 1997).

2. Ca^{2+}-Dependent Reactions

α Toxin generates Ca^{2+}-permissive pores when applied to certain cells, such as endothelial cells (Suttorp et al. 1985) and platelets (Bhakdi et al. 1988). In other cells, such as monocytes and keratinocytes, low toxin concentrations generate smaller pores that are not Ca^{2+}-permissive. At higher doses, however, Ca^{2+}-influx also occurs. In no instance are large membrane defects created such that cytoplasmic proteins egress from the cells. Therefore, Ca^{2+}-dependent machineries remain intact and are vulnerable to flooding by extracellular Ca^{2+}. Various consequences, including secretion, activation of phospholipases (Seeger et al. 1984; Suttorp et al. 1985), contraction of cytoskeletal elements (Suttorp et al. 1988), and the stimulation of constitutive nitric-oxide (NO) synthase follow (Suttorp et al. 1993). By stimulating secretion of pro-coagulatory substances from platelets, α toxin exerts potent pro-coagulatory effects (Fig. 5; Bhakdi et al. 1988). By activating the calcium-dependent phospholipase A_2, α toxin provokes generation of biologically active lipid mediators, which could contribute to inflammatory responses (Fig. 6; Seeger et al. 1984; Suttorp et al. 1985). Calcium-influx into endothelial cells provokes cytoskeletal contraction, and this leads to rounding up of the cells and formation of intercellular gaps that permit rapid leakage of macromolecules across a con-

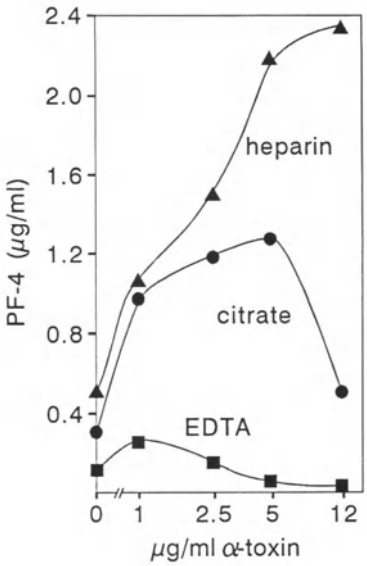

Fig. 5. Release of PF-4 from platelets in whole human blood anticoagulated with heparin (3 U/ml), citrate (10 mM), or ethylene diamine tetraacetic acid (10 mM) upon treatment with subhemolytic doses of staphylococcal α toxin. PF-4 was quantified in the supernatants after a 20-min incubation with the toxin. Massive secretion of PF-4 occurred in a calcium-dependent fashion

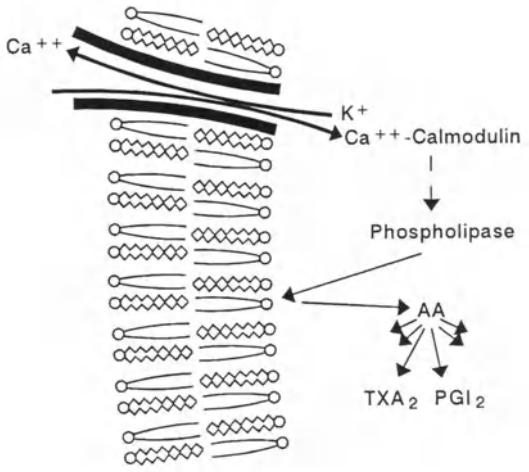

Fig. 6. A staphylococcal α-toxin-created transmembrane pore serves as a nonphysiological calcium channel. Incoming calcium, possibly after binding to calmodulin, activates phospholipases, with subsequent cleavage of arachidonic acid and formation of tissue-specific arachidonic-acid metabolites. *TXA_2*, thromboxane A_2; *PGI_2*, prostacyclin

fluent monolayer (Suttorp et al. 1988). This could be a significant event underlying development of pulmonary edema during staphylococcal pneumonia. Detrimental effects on the microcirculation could also be provoked by uncontrolled production of NO in endothelial cells, caused by Ca^{2+}-dependent triggering of constitutive NO synthase.

In addition to these secondary effects, liberation of biologically active substances from dead or dying cells may also have long-term effects. For example, keratinocytes killed by α toxin would release their content of intracellularly stored IL-1α, which would trigger and sustain inflammatory reactions.

3. Long-Range Effects of α Toxin

α Toxin can provoke acute organ dysfunction and is lethal in experimental animals (MCCARTNEY and ARBUTHNOTT 1978). In this regard, α toxin is not less potent than endotoxins. When applied intravascularly, α toxin provokes profound pathophysiological alterations in the pulmonary microvasculature, subnanomolar concentrations leading to the development of lethal pulmonary edema (SEEGER et al. 1984, 1990). The underlying mechanisms are complex and encompass the direct toxic action on endothelial cells and the production of mediators that, in turn, promote pulmonary arterial hypertension. Systemic application of α toxin in monkeys causes the animal to succumb, with symptoms that may be explained from the in vitro observations. In addition to development of pulmonary edema, a profound thrombocytopenia is observed, caused by the action of α toxin on platelets (BHAKDI et al. 1989a). Human patients suffering from severe staphylococcal infections (septicemia, burn-wound infections, pneumonia) all present with symptoms that can be partially explained by the local and systemic action of α toxin.

4. Synergism Between α Toxin and Other Toxins

An important recognition emerging in recent years is that different toxins can synergize with each other to enhance their detrimental effects in the host macro-organism. For example, priming of pulmonary cells in isolated lungs with endotoxin potentiates the vascular abnormalities in response to α toxin (WALMRATH et al. 1994). Such synergism and the resulting vascular abnormalities are probably relevant to the pathogenesis of organ failure in systemic infections.

F. Resistance and Repair Mechanisms

Resistance to toxin attack may be due to various causes. The first is the absence of high-affinity binding sites. This is exemplified by human erythrocytes, which escape attack when α toxin is present even at comparatively high concentrations (10 μg/ml). This type of resistance can be overcome by increasing toxin doses to 30–100 μg/ml, since non-specific, absorptive binding occurs (HILDEBRAND et al. 1991). It is notable that no marked differences have yet been observed between pores formed at low and high toxin concentrations, although, as alluded to above, it is possible that small variations do exist regarding stoichiometry and pore size.

A second cause of resistance is that insertion of the pore-forming sequence may be inhibited despite assembly of oligomers. This is the case with human granulocytes. Thus, α toxin binds to these cells as efficiently as it does to highly susceptible lymphocytes. Granulocyte-bound toxin oligomers form SDS-stable pre-pores that remain associated at the cell surface for surprisingly long periods (≥1 h). It is intriguing that very little endocytosis or shedding occurs. However, membrane permeabilization does not take place (Fig. 7), and this has been related by spectroscopic studies to the inability of the oligomers to insert their pore-forming sequences into the membrane (Fig. 8; VALEVA et al. 1997b). The mechanisms underlying inhibition of membrane insertion have not been clarified.

A third reason for relative resistance of cells to the lethal action of α toxin is their capacity to repair the lesions. This is exemplified by the reaction of fibroblasts. Here, α toxin binds efficiently to and permeabilizes the cells, provoking initial K^+ efflux and depletion of cellular adenosine triphosphate (ATP; WALEV et al. 1994). The latter may be due to consumption rather than to direct egress of the nucleotide through the toxin pores. After approximately 2 h, the cells start to recuperate and replenish their K^+ and ATP content. After overnight culture, no residual signs of toxin attack remain, although approximately 50% of the toxin oligomers remain detectable at the cell surface. Utilization of labeled cysteine substitution α-toxin mutants has generated

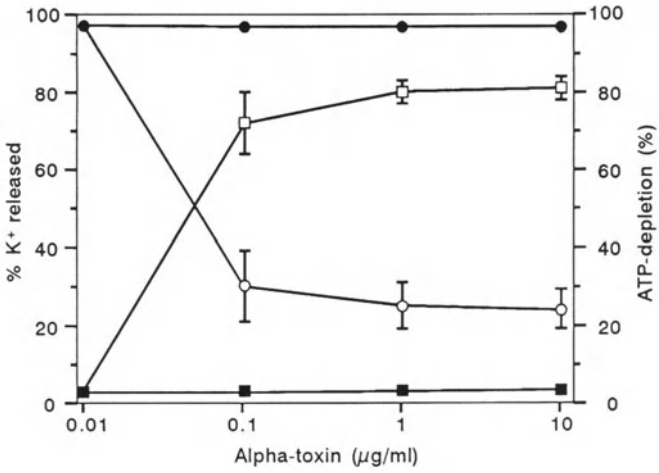

Fig. 7. Granulocytes are resistant to the permeabilizing effects of α toxin. Measurement of cellular adenosine triphosphate (ATP) depletion and K^+ efflux in α-toxin-treated cells. Lymphocytes (*open symbols*) or granulocytes (*filled symbols*) were treated with α toxin for 60 min at 37°C. The K^+ concentrations (*squares*) of supernatants were determined. Cells were lysed with Triton-X-100, and ATP concentrations in the lysates (*circles*) were determined. Data represent means±standard deviations (*error bars*) for three independent experiments

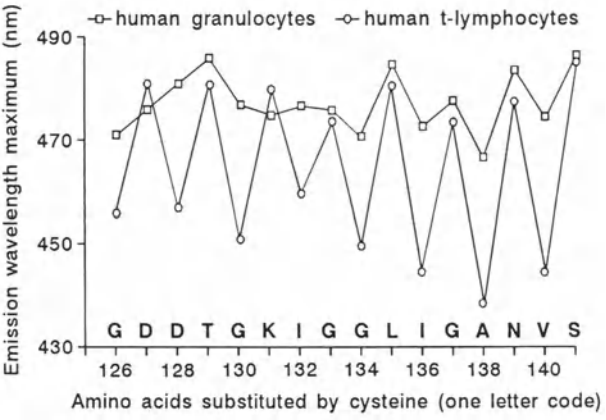

Fig. 8. Emission maxima of acrylodan-labeled α toxin cysteine mutants measured on human granulocytes and human T-lymphocytes, showing lack of lipid contact of the pore-forming domain in the toxin-resistant granulocytes

information on the conformation of the α-toxin oligomers in the repaired lesions. After repair, the amino acid side chains originally in contact with lipid continue to display blue fluorescence, indicative of their retention within the membrane. Remarkably, fluorescence of acrylodan attached to side chains in the pore lumen shifts from green to blue fluorescence. This would best be explained by exclusion of water from the pore, and a speculative model emerges in which the channel is closed through constriction. Pore closure is likely to be a process of widespread relevance in the context of attack by and defense against pore-forming proteins. The underlying molecular mechanism remains to be elucidated.

G. Use of α Toxin as a Tool in Cell Biology

α Toxin was the first pore-forming cytolysin to be exploited as a tool for selective permeabilization of plasma cell membranes (Ahnert-Hilger et al. 1985). Permeabilized chromaffin cells were shown to retain the capacity to secrete granular constituents upon stimulation with low concentrations of Ca^{2+}. Since then, an ever-increasing number of investigators are confirming that pore-forming toxins are superior tools for membrane permeabilization to conventional agents, such as digitonin or saponin. The assets of pore-forming toxins are multiple (Bhakdi et al. 1993). The actions of these agents are well-characterized and easy to control. Since α-toxin pores do not permit passage of proteins, guarantee is given that channels are exclusively formed in the plasma membrane. α-Toxin pores are quite well-defined in size, so that stable membrane lesions of approximately 1 nm diameter can be produced. It is possible to manipulate the intracellular ionic milieu and to introduce Ca^{2+} and

small molecules, such as nucleotides, into the cells. Since pure preparations of α toxin contain no enzymatic activity, there will be no proteolytic alterations of cell constituents.

Native α toxin contains no cysteines, and single-cysteine substitution mutants can be produced that usually remain functional. These substitution mutants have played a major role as tools for the elucidation of structure–function relationships during pore assembly (Valeva et al. 1995, 1996, 1997a, 1997b). Mutants containing histidine replacements (Jursch et al. 1994; Menzies and Kernodle 1994) or with nicks and overlaps in the pore-forming domain (Walker et al. 1993, 1994, 1995; Walker and Bayley 1994) have also proved valuable and harbor the potential of becoming useful tools in cell biology (Bhakdi et al. 1993; Bayley 1994). The utility of α toxin may be broadened through the availability of manipulatable toxin derivatives. Thus, it is now possible to assemble oligomers that can be opened and closed (Bayley 1994; Walker et al. 1994a, 1994b, 1995; Chang et al. 1995; Valeva et al. 1996; Russo et al. 1997). In addition to its application as a permeabilizing agent, α toxin may also become useful for the study of the turnover and dynamics of membrane microdomains, since it can be derivatized and stably labeled for use as a non-toxic membrane marker.

H. Medical Relevance of α Toxin

Although it is presently not possible to unequivocally prove that α toxin represents a microbial virulence factor in humans, all available data do clearly support this contention. The major arguments are: (1) many human cells, including monocytes, lymphocytes, endothelial cells, keratinocytes, and platelets, are effectively attacked by the toxin under physiological conditions; and (2) damage to these cells triggers pathological sequelae. These processes have been studied in detail in vitro and are mirrored by characteristic clinical presentations. Thus, homeostasis disturbances, thrombocytopenia, and pulmonary lesions are frequently encountered in patients during severe staphylococcal infections. The clinical presentation of patients with *S. aureus* skin infections can also be easily explained by the actions of α toxin on keratinocytes, macrophages, and fibroblasts. In all instances, the possibility that α toxin synergizes with other virulence factors is imminent.

A theoretical approach to relate toxin production to clinical relevance is to quantify the level of α toxin in tissues or body fluids and attempt to make a correlation with disease. Unfortunately, α toxin avidly binds to target cells, antibodies, and lipoproteins, and quantification methods must, therefore, first solve the task of liberating the toxin from these binders. Consequently, a sensitive immunoassay for quantifying α toxin in tissues and body fluids is not yet available.

Indirect demonstration of a significant role for α toxin as a virulence factor would be possible if application of specific high-titered antibodies were found

to be therapeutically effective. Polyclonal and monoclonal human hyperimmune globulins against α toxin have been prepared, and they are able to suppress all experimental toxic effects in vitro and in vivo. Patients with early diagnosis of severe *S. aureus* infections are candidates for therapy with such hyperimmune globulins, but clinical trials with these antibodies have not yet been conducted. Construction and production of α-toxin vaccines are very simple. A single-substitution mutant has been found to be devoid of all toxic effects in experimental animals and to induce high protective-antibody levels. The feasibility of using such vaccines in veterinary medicine is presently being explored.

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CHAPTER 23

Bacterial Phospholipases

R.W. TITBALL and J.I. ROOD

A. Introduction

Pioneering work by MACFARLANE and KNIGHT showed for the first time that a bacterial toxin – the α-toxin of *Clostridium perfringens* – also had enzymatic activity as a phospholipase C (PLC; MACFARLANE and KNIGHT 1941). This finding stimulated great interest not only in the α-toxin but also in the potentially toxic activity of PLC enzymes produced by other bacteria and in the mechanisms that control the production of these proteins. In addition to the PLCs, bacteria have been shown to produce other phospholipases, such as phospholipase Ds (PLDs) and phospholipase As (PLAs), which differ in the site of phospholipid cleavage (Fig. 1). Today, many phospholipases, produced by both pathogenic and non-pathogenic bacteria, have been described and characterised, but only a few of these enzymes have been shown to be lethal toxins. Nevertheless, some of the enzymes considered to be non-toxic have been shown to play important roles in the pathogenesis of disease. Recent studies have suggested that lethality is a rather crude indicator of the potential roles of these enzymes in disease and that many of these phospholipases appear to exert their effects by allowing the bacteria to colonise the host or by perturbing the metabolism of host cells rather than by directly damaging the host (TITBALL 1993; SONGER 1997). These findings have challenged the conventional criteria by which a bacterial product is considered to be a toxin. The aim of this chapter is to review the broad spectrum of bacterial phospholipases, to discuss the mechanisms by which the expression of phospholipase-encoding genes are regulated, to examine the ways in which these enzymes interact with host cells at a molecular level and to illustrate the ways in which bacterial phospholipases contribute to the pathogenesis of disease.

B. Related Groups of Phospholipases

Prior to the availability of amino-acid-sequence data, the bacterial phospholipases were grouped according to the site of cleavage of phospholipids – i.e. as PLAs, PLCs or PLDs (Fig. 1). However, there were indications that some of the bacterial enzymes within these groups are related. For example, antisera against the *C. perfringens* α-toxin was shown to cross-react with the *C.*

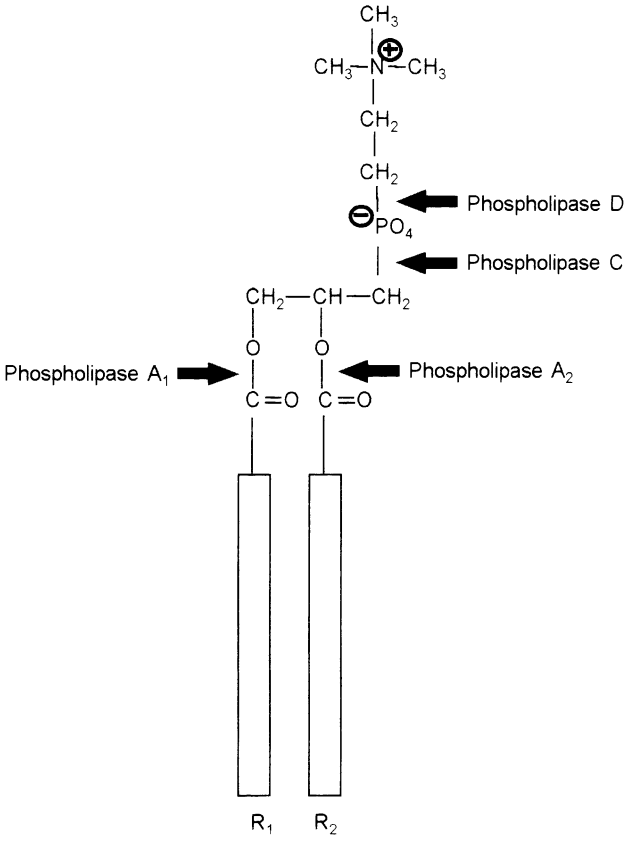

Fig. 1. Site of cleavage of phosphatidylcholine by phospholipases A, C and D. The fatty-acyl tail groups of the phospholipid are depicted as R_1 and R_2 groups

bifermentans enzyme and with the *C. novyi* γ-toxin. Over the past decade, our increased knowledge of the molecular biology of the bacterial phospholipases has allowed this structural relationship to be explored and has revealed that many of the bacterial enzymes are related and can be grouped according to both the site of phospholipid cleavage and the amino-acid-sequence homology (Table 1). Not all of these groups of phospholipases will be covered in this chapter; rather, we have selected the best-characterised groups of enzymes for further discussion.

I. Zinc Metallophospholipase Cs

The zinc metallophospholipase Cs are typified by the *C. perfringens* α-toxin and the *Bacillus cereus* phosphatidylcholine-preferring PLC (PC-PLC). Crystallographic studies (HOUGH et al. 1989; NAYLOR et al. 1998) indicate that these

Table 1. Representative bacterial phospholipases. References cited are for the cloning and nucleotide sequencing of encoding genes or for the analysis of purified enzyme

Source of enzyme	Name mass (Da)	Molecular	Substrate specificity	Ion requirements	Haemolysis
PLAs					
Helicobacter pylori	PLA_2[a]	NR	PC	NR	NR
Vibrio parahaemolyticus	Thermolabile haemolysin[b]	45 308	PC, LPC	NR	+
Zinc metallophospholipase Cs					
Clostridium perfringens	α-Toxin[c]	42 523	PC, SPM, PS, LPC	Zn^{2+}, Ca^{2+}	+
C. bifermentans	PLC[d]	42 776	NR	Ca^{2+}	±
C. novyi	γ-Toxin[e]	42 562	PC, SPM, LPC, PE, PS, PG	Zn^{2+}, Ca^{2+}, Mg^{2+}	+
C. paraperfringens	PLC[f]	NR	NR	NR	
C. absonum	PLC[f]	NR	NR	NR	NR
Bacillus cereus	PC-PLC[g]	28 520	PC, PE, PS	Zn^{2+}	
Listeria monocytogenes	PLC-B[h]	27 709	PC, PE, PS, SPM	Zn^{2+}	±
Pseudomonas fluorescens	PLC[i]	39 500	PC, PE, PS	Zn^{2+}, Ca^{2+}	NR
Gram-negative PLCs					
P. aeruginosa	PLC-H[j]	78 352	PC, SPM, LPC	NR	+
	PLC-N[j]	73 455	PC, PS	NR	
Burkholderia cepacia	PLC[k]	72 000	PC, SPM	NR	+
Burkholderia pseudomallei	PLC[l]	NR	NR	NR	+
Mycobacterium tuberculosis	MPC-A[m]	56 103	PC, SPM	NR	NR
	MPC-B[m]	56 137	PC, SPM	NR	NR
Other PLCs					
Ureaplasma urealyticum	PLC[n]	NR	pNPPC	NR	NR
Legionella pneumophila	PLC[o]	50 000–54 000	PC	NR	
V. cholerae	PL[p]	47 600	NR	NR	NR
Sphingomelinase Cs					
B. cereus	Smase[q]	34 233	SPM	Mg^{2+}	h
Leptospira interrogans	Smase[r]	41 200	SPM, PC	Mg^{2+}	h
Staphylococcus aureus	β-toxin[s]	34 546	SPM, LPC	Mg^{2+}	h
PI PLCs					
C. novyi	PI-PLC[t]	30 000	PI	NR	
B. cereus	PI-PLC[u]	34 466	PI, LPI	NR	

Table 1. *Continued*

Source of enzyme	Name mass (Da)	Molecular	Substrate specificity	Ion requirements	Haemolysis
Bacillus thuringiensis	PI-PLC[v]	34 515	PI, LPI	NR	
L. monocytogenes	PLC-A[w]	33 532	PI	NR	
Phospholipase Ds					
Corynebacterium pseudotuberculosis	PLD[x]	31 109	LPC, SPM	NR	c
Arcanobacterium haemolyticum	PLD[y]	31 977	LPC, SPM	NR	c
V. damsela	PLD[z]	69 000	PC, PE, SPM	NR	+

C, haemolytic in the presence of cholesterol oxidase from *Rhodococcus equi*; *h*, hot cold haemolysis; *LPC*, lysophosphatidylcholine; *LPI*, lysophosphatidylinositol; *MPC*, metallophospholipase C; *NR*, not reported; *PC*, phosphatidylcholine; *PE*, phosphatidylethanolamine; *PG*, phosphatidylglycerol; *PI*, phosphatidylinositol; *PLA*, phospholipase A; *PLC*, phospholipase C; *PLD*, phospholipase D; *pNPPC*, *p*-nitrophenylphosphorylcholine; *PS*, phosphatidylserine; *SPM*, sphingomyelin.

[a] Ottlecz (1993).
[b] Shinoda et al. (1991).
[c] Titball et al. (1989).
[d] Tso and Siebel (1989).
[e] Tsutsui et al. (1995).
[f] Nakamura et al. (1973).
[g] Johansen et al. (1988).
[h] Vazquez-Boland et al. (1992).
[i] Crevel et al. (1994).
[j] Ostroff et al. (1990).
[k] Vasil et al. (1990).
[l] Mack (personal communication).
[m] Johansen et al. (1996); Leao et al. (1995).
[n] De Silva and Quinn (1987).
[o] Baine (1988).
[p] Fiore et al. (1997).
[q] Yamada et al. (1988).
[r] Segers et al. (1990).
[s] Projan et al. (1989).
[t] Taguchi et al. (1980).
[u] Kuppe et al. (1989).
[v] Henner et al. (1988).
[w] Leimeister-Wächter et al. (1991).
[x] McNamara et al. (1994).
[y] Cuevas and Songer (1993).
[z] Kreger et al. (1987).

Table 2. Biological activities of site-directed mutants of *C. perfringens* α-toxin

Mutation	Domain	Proposed function of target amino acid	Activity				Bound zinc (mol)
			pc	sph	hly	let	
Wild type			+	+	+	+	2
EDTA-treated wild type			–	ND	ND	ND	2
W1S[a]	N	Zn^{2+} binding	–	–	–	–	ND
H11S[a]	*N*	Zn^{2+} binding	–	–	–	ND	ND
H68S[a]	*N*	Zn^{2+} binding	–	–	–	ND	ND
H68G[b]	*N*	Zn^{2+} binding	–	–	–	–	2
H126S[a]	*N*	Zn^{2+} binding	–	–	–	ND	ND
H126G[b]	*N*	Zn^{2+} binding	–	–	–	–	2
H136S[a]	*N*	Zn^{2+} binding	–	–	–	ND	ND
H136G[b]	*N*	Zn^{2+} binding	–	–	–	–	2
H136A[b]	*N*	Zn^{2+} binding	–	–	–	–	ND
H148S[a]	*N*	Zn^{2+} binding	–	–	–	ND	ND
H148G[b]	*N*	Zn^{2+} binding	–	–	–	–	1
H148L[b]	*N*	Zn^{2+} binding	–	–	–	–	ND
E152D[c]	*N*	Zn^{2+} binding	–	–	–	ND	1.5
E152Q[c]	*N*	Zn^{2+} binding	–	–	–	ND	1
E152G[c]	*N*	Zn^{2+} binding	–	–	–	ND	1

+, greater than 85% retention of the activity of the wild-type toxin; –, less than 3% retention of activity of the wild type toxin; *EDTA*, ethylene diamine tetraacetic acid; *ND*, not determined.
[a] Guillouard et al. 1996.
[b] Nagahama et al. 1995.
[c] Nagahama et al. 1997.

proteins contain three zinc ions which form part of the putative active sites. However, one of these ions is relatively loosely bound, which might explain why atomic-absorption spectroscopy indicates that the α-toxin contains only two zinc ions (Table 2). It is likely that treatment of the proteins with chelating agents, such as ethylenediaminetetraacetic acid and *o*-phenanthroline, with the resultant PLC inactivation, also removes this zinc ion (Krug and Kent 1984; Hough et al. 1989; Titball and Rubidge 1990). Crystallographic data indicate that the zinc ions are coordinated by histidine, tryptophan and glutamic acid side chains in an α-helical domain (Hough et al. 1989; Naylor et al. 1998). Confirmation of the roles of these amino acid side chains has been obtained by using site-directed mutagenesis to construct mutant forms of the *C. perfringens* α-toxin (Table 2). These mutants were all devoid of PLC activity toward PC and sphingomyelin, were devoid of haemolytic activity and, for those mutants which were evaluated, were non-toxic. This finding suggests that all of these activities are dependent on a single active site. The amino acids that are involved in zinc-ion coordination are conserved in the *C. novyi* γ-toxin (Tsutsui et al. 1995) and in the *C. bifermentans* PLC (Tso and Siebel 1989),

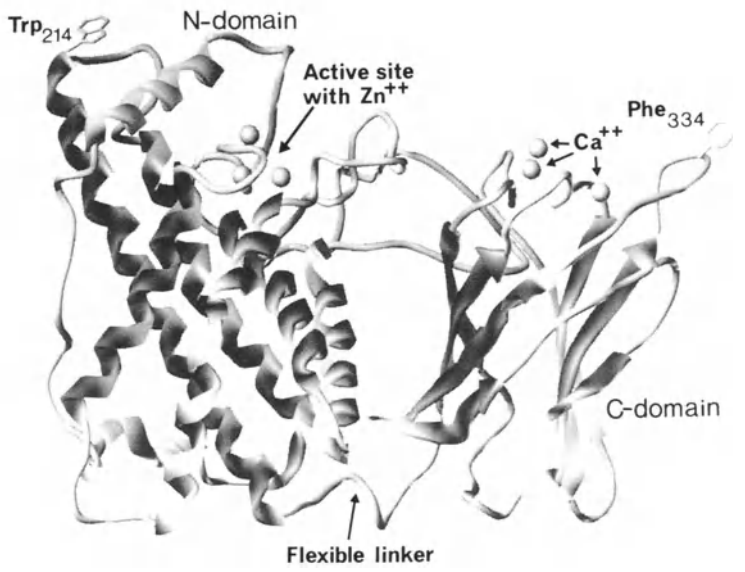

Fig. 2. Cartoon trace of the *Clostridium perfringens* α-toxin, showing the locations of zinc ions, calcium ions and exposed hydrophobic amino acids, which might play a role in membrane insertion

suggesting that the active sites of these enzymes also contain three zinc ions. The other members of the zinc-metallophospholipase-C group (*C. absonum* PLC and *C. barati* PLC; Nakamura et al. 1973) have been assigned on the basis of immunological cross-reactivity with the *C. perfringens* α-toxin and/or the ability of zinc-chelating compounds to inactivate these enzymes.

Although the active-site regions of the different zinc metallophospholipase Cs may be similar, these enzymes differ in that some are single-domain proteins whilst others possess an additional C-terminal domain (Titball 1993; Titball et al. 1993). This difference in molecular organisation has been investigated by comparing the crystal structure of *C. perfringens* α-toxin (Fig. 2) with that of the *B. cereus* PC-PLC (Naylor et al. 1998). The N-terminal domain (the active-site domain) of the α-toxin shows an overall structural similarity to the entire *B. cereus* PC-PLC (Titball et al. 1991; Naylor et al. 1998). The additional domain of the *C. perfringens* α-toxin is in a β-sheet conformation and lies adjacent to the active site. One key biological difference between the *C. perfringens* α-toxin and the *B. cereus* PC-PLC is that the *C. perfringens* enzyme is toxic and is able to lyse erythrocytes, suggesting that the C-domain plays a key role in this process. In this context, it should be noted that the other zinc metallophospholipase Cs that have a C-domain (*C. novyi* γ-toxin and *C. bifermentans* PLC) are also haemolytic.

II. Gram-Negative PLCs

Most studies with enzymes in the gram-negative-PLC group have been carried out with PLCs from *P. aeruginosa*. Two related enzymes (PLC-N and PLC-H; Ostroff et al. 1990) are produced by this bacterium. Both are able to hydrolyse PC, but only PLC-H is able to hydrolyse sphingomyelin and is haemolytic (Ostroff et al. 1990). Little is known about the molecular organisation of these enzymes, although it appears that the PLC-H protein is post-translationally modified to form the active enzyme by another *P. aeruginosa* protein, PlcR, which is encoded downstream of *plcH* (Shen et al. 1987)

The enzymes produced by *P. aeruginosa* are now known to be closely related to the PLCs produced by a number of other bacteria, including *Mycobacterium tuberculosis* (Johansen et al. 1996; Leao et al. 1995), *Burkholderia pseudomallei* (Mack, personal communication) and *B. cepacia* (Vasil et al. 1990). Although the *Burholderia* enzymes are similar in both size and amino acid sequence to the *Pseudomonas* enzymes, the mycobacterial enzymes (MpcA and MpcB) lack a 20-kDa C-terminal region. Another major difference between these enzymes is that the *Pseudomonas* and *Burkholderia* enzymes are exported from the cell, whilst the mycobacterial enzymes appear to be cell associated, which might explain why *M. tuberculosis* has a contact-dependent haemolytic activity (Leao et al. 1995). The reason for the difference in cellular location of the mycobacterial enzymes has yet to be explained, but it is worth noting that this bacterium is an intracellular pathogen, whilst the other bacteria in this group are extracellular pathogens.

III. Phosphatidylinositol PLCs

The PLCs that are able to specifically hydrolyse phosphatidylinositol (PI) and glycerophosphatidylinositol (GPI) are related and are produced by gram-positive bacteria. Although the proteins in this group show limited amino-acid-sequence identity (the *B. cereus* PI-PLC and the *Listeria moncytogenes* PlcA are 24% identical), the crystal structures of these enzymes are similar (Moser et al. 1997; Heinz et al. 1998), suggesting that all of the PI-PLCs have a similar architecture. The *B. cereus* PI-PLC and the *L. monocytogenes* PlcA are single-domain proteins, folded as a $(\beta\alpha)_8$-barrel, where the α-helices are located outside of an inner β-barrel (Moser et al. 1997; Heinz et al. 1998). The active sites of the enzymes are located at the C-terminal ends of the β-barrel regions.

The ability of these enzymes to hydrolyse GPI is of particular interest, since many eukaryotic cell-surface proteins are linked to GPI phospholipids, and cleavage of the PI–glycan–ethanolamine anchor results in the release of the protein from the cell surface. The treatment of various eukaryotic cells with bacterial PI-PLCs has been shown to release alkaline phosphatase, 5′ nucleotidase, alkaline phosphodiesterase and acetycholinesterase from the cell surface (Ikezawa 1986). This significance of these findings has not been fully

explored, but it seems possible that some of these enzymes might be exploited by the bacterium – for example, as components of a phosphate-scavenging pathway.

IV. Phospholipase Ds

Whilst a number of bacteria have been shown to produce PLDs, the group of enzymes that has been investigated in the most detail includes *Corynebacterium pseudotuberculosis*, *C. ulcerans* and *Arcanobacterium haemolyticum* PLDs (MCNAMARA et al. 1995). The bacterial enzymes are 64–97% identical, and antibodies against the individual proteins show some cross-neutralisation activity against the other enzymes. Intriguingly, these proteins show amino-acid-sequence homology with the PLD in brown-recluse-spider venom, especially at the N-terminus (TRUETT and KING 1993; SONGER 1997). The bacterial enzymes preferentially hydrolyse sphingomyelin and lysophosphatidylcholine and cause haemolysis of erythrocytes only in the presence of the *Rhodococcus equi* cholesterol oxidase. In contrast, treatment of erythrocytes with *C. pseudotuberculosis* PLD renders them resistant to lysis with *Staphylococcus aureus* sphingomyelinase (β-toxin). Not all bacterial PLDs behave in this way. The enzyme produced by *Vibrio vulnificus* is able to hydrolyse sphingomyelin and PC and is haemolytic (KOTHARY and KREGER 1985; KREGER et al. 1987), and it seems likely that this enzyme is a member of a distinct group of bacterial PLDs.

C. Functional and Biological Properties of Phospholipases

By definition, phospholipases are able to hydrolyse phospholipids, with two main types of phospholipid able to serve as substrates. All phospholipids possess a water-soluble head group linked to a phosphate group and to hydrophobic fatty-acyl tails. The nature of the head group can be quite variable, ranging from charged groups, such as choline, to inositol sugars (Fig. 3). Glycerophospholipids, such as PC, phosphatidylserine, PI and phosphatidylglycerol, contain a glycerol backbone linking the fatty-acyl tails to the head group. Sphingolipids (sphingomyelins) contain a ceramide moiety (the backbone and fatty-acyl tail) linked to the head group.

The mechanisms by which phospholipases recognise their substrates are not well understood. However, the crystal structures of the *B. cereus* PC-PLC (HOUGH et al. 1989) and PI-PLC (HEINZ et al. 1998), the *C. perfringens* α-toxin (NAYLOR et al. 1998) and the *L. monocytogenes* PlcA (MOSER et al. 1997) all reveal an active-site cleft, which is thought to accommodate the head group of the phospholipid. The variations in the size and charge of the head group might, therefore, be important in determining the specificity of the different enzymes. The active site of the *B. cereus* PI-PLC and the *L. monocytogenes* PlcA forms a wide cleft, into which the inositol head group binds in an edge-

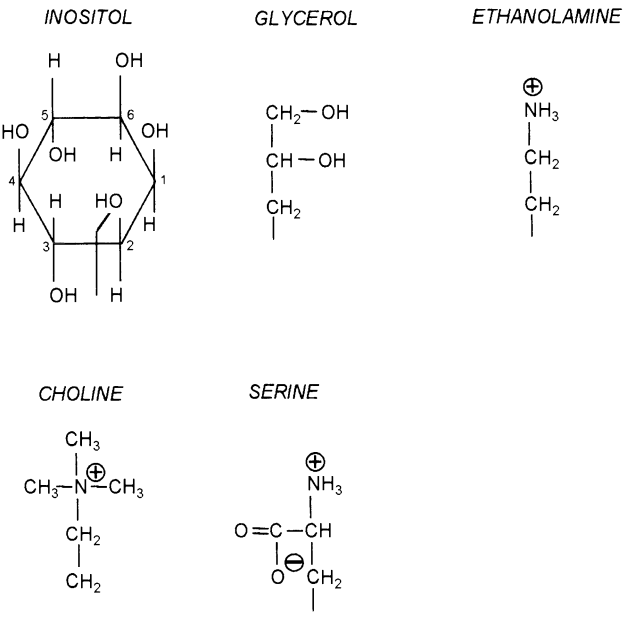

Fig. 3. Examples of phospholipid-head groups

on mode (Moser et al. 1997; Heinz et al. 1998). A number of hydrogen bonds are formed between the inositol head group and the side chains of amino acids lining the active site. As might be expected, the hydroxyl groups at positions 1 and 6 of the inositol ring are accessible to the solvent, and these groups are linked to other groups in the natural phospholipid substrate.

For the *B. cereus* PC-PLC and the *C. perfringens* α-toxin, the active-site cleft is much narrower (Hough et al. 1989; Naylor et al. 1998), which is in accordance with the ability of these enzymes to hydrolyse phospholipids with less bulky head groups; indeed, these enzymes are unable to hydrolyse PI substrates. However, in spite of this constraint on the size of the head group, these enzymes are able to hydrolyse a remarkably wide range of substrates. For example, the *B. cereus* PC-PLC is reported to be able to hydrolyse PC, phosphatidylserine and phosphatidylethanolamine, which suggests that only conserved regions of the different head groups are recognised by these phospholipases. This suggestion is, to some extent, supported by experimental findings that the phosphate moiety of the substrate forms a strong network of hydrogen bonds with the three zinc ions, which are found in the active site of the protein (Hansen et al. 1993a, 1993b). The head group also forms hydrogen bonds with the side chains of amino acids lining the active site, and this might explain why the enzyme has subtly different activities towards different substrates.

In eukaryotes, most of the phospholipid is found in cell membranes, where it forms a water-impermeable bilayer, with the hydrophobic tail groups embedded within the membrane. The phospholipid bilayer also provides a matrix for the display of various biologically active proteins and carbohydrates. However, membrane phospholipids perform more than a passive role in the maintenance of cell function. The products of phospholipid hydrolysis, especially the fatty-acyl tail groups, are key signalling molecules that modulate cellular metabolism. Perhaps it is not surprising that many bacterial phospholipases are thought to modulate cell metabolism via the hydrolysis of phospholipids and the generation of secondary messenger molecules. These effects are discussed in more detail below.

D. Modulation of Eukaryotic Cell Metabolism

I. Hydrolysis of Membrane Phospholipids

Many of the bacterial phospholipases that play important roles in the pathogenesis of disease are active towards eukaryotic cells. Erythrocytes are often used for these studies, because the effect of the enzyme can easily be measured in the laboratory as haemolysis. The lysis of these cells is thought to be due to the degradation of membrane phospholipids, which leads to weakening of the cell membrane and eventual lysis (Titball et al. 1993). Although phospholipids in erythrocyte membranes are degraded after treatment with phospholipases (Colley et al. 1973; Sakurai et al. 1993, 1994), it not certain that the direct action of the bacterial enzyme on membrane phospholipids is responsible for cell lysis. One possibility is that the action of the bacterial enzyme is to cause activation of endogenous membrane phospholipases, which are then responsible for much of the membrane damage. Evidence supporting this suggestion has been provided by Sakurai et al. (1993, 1994) who showed that membrane phospholipases C and D are activated in rabbit erythrocyte membranes treated with α-toxin and that inhibitors of the PLC activity of α-toxin (neomycin and 2-nitro-4-carboxyphenyl-*N*,*N*-diphenylcarbamate) do not inhibit haemolytic activity of the α-toxin. Another suggestion is that the limited hydrolysis of the membrane phospholipids leads to the accumulation of the hydrophobic fatty-acyl groups within the central region of the membrane bilayer, and that the resultant stresses in the membrane result in cell lysis (Bowman et al. 1971; Colley et al. 1973).

Several workers have suggested that the haemolytic activity of phospholipases indicates their toxicity and their likely role in the pathogenesis of disease (MacFarlane 1955; Ostroff et al. 1990; Titball 1993; Songer 1997). However, it is difficult to define what is meant by a non-haemolytic phospholipase, since the activity of haemolytic enzymes towards erythrocytes from different species (Jolivet-Reynaud et al. 1988), towards erythrocytes suspended in different buffers and even towards erythrocytes of different ages (Little et al. 1981) varies. However, it is generally accepted that phospholipases, which

Table 3. The relationship between haemolytic activity and the lateral pressure at which the hydrolysis of phospholipids ceases in a phospholipid monolayer (Möllby et al. 1978)

Source of enzyme	Type	Haemolysis of human erythrocytes	Maximum lateral pressure at which hydrolysis ceases (dyne/cm)
Pig pancreas	A_2		16.5
Cabbage	D		20.5
Crotalus adamanteus venom	A_2		23
Bacillus cereus PC-PLC	C		31
Naja naja venom	A_2	+	34.8
Bee venom	A_2	+	35.3
Staphylococcus aureus	C	+	>40
Clostridium perfringens	C	+	>40

PC-PLC, phosphatidylcholine phospholipase C.

play important roles in disease, are able to interact with membrane phospholipids, and this might cause lysis of cells.

The insertion of phospholipases into lipid monolayers was studied by Van Deenen et al. (1976), who showed that, by increasing the lateral pressure within a phospholipid monolayer, it was possible to identify two groups of enzymes (Table 3). The enzymes in one group were able to hydrolyse the phospholipid in the monolayer at lateral pressures above those found in typical membranes (31–35 dyne/cm; Mollby 1978), and enzymes in this group were also haemolytic – perhaps a reflection of their ability to be inserted into membrane bilayers.

Only recently have we gained some insight into the molecular mechanisms by which these enzymes interact with membranes. In the case of the *C. perfringens* α-toxin, the crystal structure of the protein has revealed that the protein possesses surface-exposed hydrophobic amino acid side chains (Trp_{214}, Tyr_{331} and Phe_{334}) that are positioned on the active-site faces of the N- and C-domains of the protein in a way that enables them to interact with the hydrophobic tail groups of membrane phospholipids (Naylor et al. 1998). In addition, the C-domain contains a partial calcium-binding site (Guillouard et al. 1997), and a model of the α-toxin docked with a membrane suggests that the phosphate head groups of membrane phospholipids provide the additional calcium-coordinating ligands (Naylor et al. 1998). The C-domain of the α-toxin shows significant structural homology with calcium-dependent phospholipid-binding domains from mammalian enzymes (Fig. 4), such as PI-PLC, arachidonate-5-lipoxygenase and synaptotagmin (Guillouard et al. 1997; Naylor et al. 1998). This model is supported by the long-standing observation that calcium ions are absolutely required for the activity of the α-toxin (Krug and Kent 1984; Moreau et al. 1988; Nagahama et al. 1996) and that calcium ions have not been detected in purified preparations of the α-toxin (Krug and

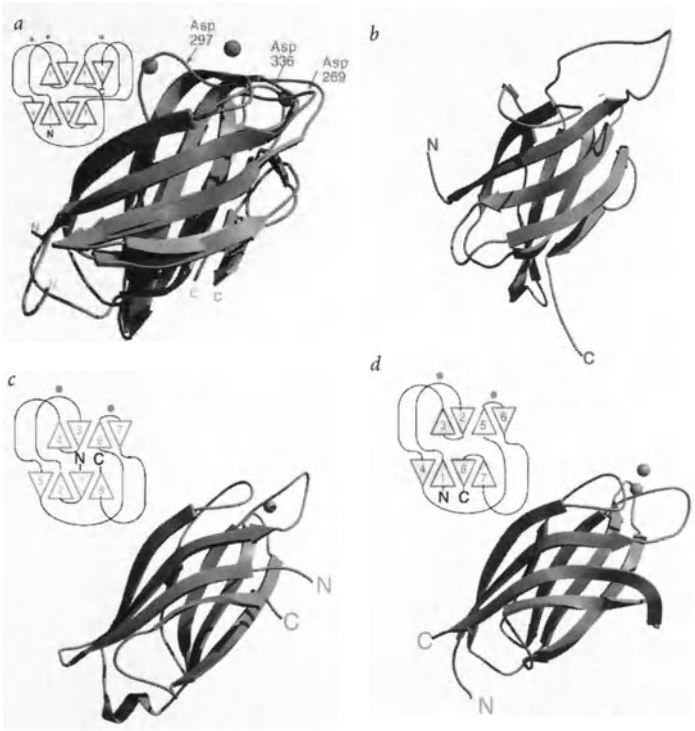

Fig. 4. Structural similarity of the C-terminal domain of α-toxin (**a**) with the lipid-binding domains of soybean lipoxygenase 1 (**b**), synaptotagmin (**c**) and mammalian phosphoinositide-specific phospholipase C Cδ1 (**d**). NAYLOR et al. (1998)

KENT 1984), probably because of the low affinity of the α-toxin for calcium ions in the absence of phospholipid (GUILLOUARD et al. 1997). The model is also supported by a comparison with the crystal structure of the non-haemolytic *B. cereus* PC-PLC, which lacks a C-domain (and, therefore, the calcium-binding site) and also has amino acids without hydrophobic side chains at comparable positions in the α-toxin (NAYLOR et al. 1998; HOUGH et al. 1989).

The mechanism by which other phospholipases interact with membrane phospholipids are less well understood, and many of these enzymes do not require divalent cations, such as calcium, to facilitate the interaction. Nevertheless, it seems likely that at least partial insertion of the enzyme into the phospholipid bilayer is required. The active site of the *B. cereus* PI-PLC is surrounded by a ridge of hydrophobic amino acids, and it is proposed that this hydrophobic surface (which has an area of ~350 $Å^2$) becomes inserted into the membrane (HEINZ et al. 1998).

II. Hydrolysis of Membrane Phospholipids Modulates Cell Metabolism

Many of the early studies with bacterial phospholipases considered that haemolysis was the key indicator of activity, and it was suggested that the haemolytic enzymes produced by pathogens might function by causing lysis of host cells. In fact widespread haemolysis is rarely, if ever, encountered in cases of disease caused by pathogens that produce phospholipases (HÜBL et al. 1993). Nevertheless, phospholipases do play important and, in some cases, key roles in the pathogenesis of disease, and recent studies suggest that these enzymes function not by causing wide-scale cell lysis but by causing limited hydrolysis of membrane phospholipids, the products of which modulate cell metabolism to the detriment of the host and to the benefit of the pathogen.

Central to this process is the generation of diacylglycerol from glycerophospholipids and ceramide from sphingolipids. Diacylglycerol affects cell metabolism in several ways. First, it is able to serve as a substrate for diacylglycerol lipase, and the product of this enzyme (arachidonic acid) can, in turn, serve as a substrate for the arachidonic-acid pathway (SAMUELSSON 1993). The activation of the arachidonic-acid pathway has been reported in cells exposed to a variety of phospholipases, including the *C. perfringens* α-toxin (FUJII and SAKURAI 1989; GUSTAFSON et al. 1990; BUNTING et al. 1997), the *P. aeruginosa* PLC-H (KÖNIG et al. 1997) and the *B. cereus* PC-PLC (LEVINE et al. 1988). The products of the arachidonic-acid pathway include pro-inflammatory molecules, such as the prostaglandins, thromboxanes and leukotrienes, which cause vasodilation and bronchonstriction and cause mucus secretion (SAMUELSSON 1983). A more detailed analysis of the roles of these molecules is beyond the scope of this review. However, it is worth pointing out that these molecules are also central to the platelet-aggregation pathway, which might explain the long-standing observations that some phospholipases cause platelet aggregation (SUGAHARA et al. 1976; OHSAKA et al. 1978; COUTINHO et al. 1988).

Second, diaycylglycerol can activate protein kinase C (PKC) within cells, and the activated PKC is thought to activate eukaryotic membrane-bound phospholipases (WAITE 1987; EXTON 1990; NISHIZUKA 1992). These activated phospholipases would lead to further hydrolysis of membrane phospholipids, and this has lead to the suggestion that haemolysis might actually be an indication of self-degradation of membrane phospholipids rather than a direct effect of the bacterial enzyme on the membrane (SAKURAI et al. 1993, 1994; OCHI et al. 1996).

Of equal significance is the ability of the activated eukaryotic phospholipases to generate further substrates for the arachidonic-acid cascade and to further activate PKC. Hence, the initial interaction of the bacterial enzyme with the host cell membrane might trigger a positive-feedback loop, which leads to the eventual self-destruction of the cell. Of the eukaryotic phospholipases that might be activated, the PI-PLCs merit special consideration,

because the inositol triphosphate (IP_3) generated by hydrolysis of inositol diphosphate would act as a secondary messenger within the cell. One effect of IP_3 would be to cause the release of calcium from the endoplasmic reticulum, and this would, in turn, cause both the opening of calcium gates in the membrane and the contraction of muscle tissues (Sakurai et al. 1990).

E. Regulation

1. Regulation of the *C. perfringens plc* Gene

The *C. perfringens* α-toxin is encoded by the *plc* gene, which is sometimes referred to as the *cpa* gene and is located in a hyper-variable region of the *C. perfringens* chromosome (Cole and Canard 1997). Immediately upstream of the *plc* promoter, there is a series of poly-A repeat sequences that appear to affect the ability of the DNA to bend (Toyonaga et al. 1992). Deletion of this 77-bp AT-rich region increases the expression of the *plc* gene in *Escherichia coli*, suggesting that this region of DNA curvature has a negative regulatory role in α-toxin production. However, in *C. perfringens*, deletion of one or more of the dA_{5-6} tracts located within the 77-bp region leads to decreased α-toxin production (Matsushita et al. 1996). These results imply that host factors are important in *plc* gene regulation, a conclusion supported by gel-retardation studies, which have shown that, in *C. perfringens*, there is an uncharacterised factor that binds within the *plc* gene (Katayama et al. 1993).

Other studies have identified a two-component signal-transduction system which globally regulates extracellular toxin production in *C. perfringens* (Lyristis et al. 1994; Shimizu et al. 1994). Mutation of either the *virS* or *virR* genes, which encode a sensor histidine kinase and a response regulator, respectively, leads to the complete loss of θ-toxin production and reduced production of α-toxin, κ-toxin, and other extracellular enzymes. Regulation of this system occurs at the transcriptional level (Ba-Thein et al. 1996), but the precise mechanism by which the activated VirR protein controls α-toxin production is not known. The environmental factors that stimulate the VirS/VirR regulatory cascade are also unknown (Rood and Lyristis 1995).

II. Environmental Control of PLC Production in *P. aeruginosa*

The PLC-N and PLC-H PLC enzymes from *P. aeruginosa* are encoded by the *plcN* and *plcH* (or *plcS*) genes, respectively. Both genes are induced by phosphate starvation, choline or glycine betaine (Vasil et al. 1994). Activation of both *plcH* and *plcN* in an environment low in inorganic phosphate (P_i) is dependent upon a homologue of the *E. coli* PhoB protein, a response regulator which activates the transcription of the P_i regulon in *E. coli*. In *P. aeruginosa*, this *phoB* (or *plcA*) regulon includes the gene encoding alkaline phosphatase and the two phospholipases (Shortridge et al. 1992; Vasil et al. 1994).

The *plcH* gene is the first gene in an operon that also includes two in-phase overlapping genes, *plcR1* and *plcR2*, which encode proteins that appear to be required for the secretion of PlcH (but not PlcN) from *P. aeruginosa* cells (Cota-Gomez et al. 1997). The *plcHR* operon has both PhoB-dependent and PhoB-independent promoters, the latter being involved in *plcH* activation by the PLC end-product, phosphorylcholine, as well as choline and glycine betaine. Choline and glycine betaine are breakdown products of phosphorylcholine and act as osmoprotectants and potential carbon, nitrogen and energy sources. A novel catabolite-repression system appears to be involved in the osmoprotectant-dependent regulation of *plcH* transcription (Sage and Vasil 1997). These compounds also activate *plcN* which, since it only has one PhoB-dependent promoter, requires both PhoB and P_i starvation for choline- and glycine-betaine-mediated activation (Shortridge et al. 1992; Vasil et al. 1994). Choline-induced activation of *plcH* requires the *orp* gene, which encodes a putative regulatory protein, and *betB*, which encodes a betaine-aldehyde dehydrogenase, an enzyme involved in the synthesis of glycine betaine from choline (Sage et al. 1997).

III. Regulation of the *Listeria* Phospholipases by PrfA

The *Listeria* phospholipases PI-PLC and PC-PLC are encoded by the *plcA* and *plcB* genes, respectively. These genes are located within a cluster of virulence genes on the *L. monocytogenes* chromosome but are encoded on divergently transcribed operons (Fig. 5). The *plcB* gene is the third gene in an operon that includes the *mpl* gene, the product of which is a zinc metalloprotease involved in the post-translational activation of PC-PLC (Raveneau et al. 1992; Poyart et al. 1993), and the *actA* gene, which encodes a protein involved in host actin polymerisation (Smith and Portnoy 1997). Proximal to this operon is the listeriolysin-O gene, *hly*, followed by the divergently expressed *plcA* and *prfA* genes (Fig. 5). The *pfrA* gene is expressed both from

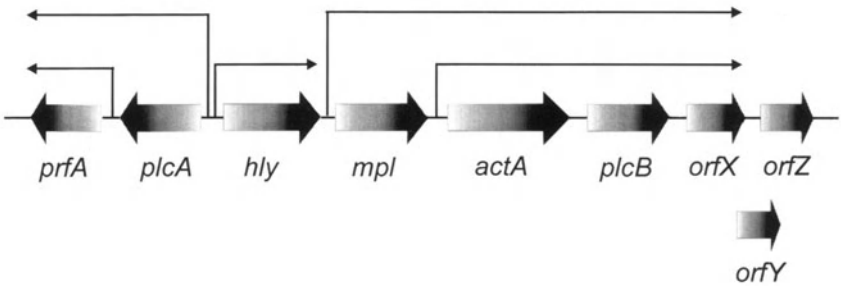

Fig. 5. Genetic organisation of the *Listeria monocytogenes* toxin gene locus. The respective genes and their transcriptional orientations are shown by the *thick arrows*. The major transcripts are shown by the *thin arrows*

its own promoter and from the *plcA* promoter (Mengaud et al. 1991). It encodes a regulatory protein that activates the transcription of all of these genes, including *plcA* and *plcB* (Mengaud et al. 1991; Chakraborty et al. 1992). It is also autoregulatory, activating its own expression from the *plcA* promoter and repressing transcription from the monocistronic *prfA* promoter (Freitag et al. 1993; Freitag and Portnoy 1994). Other *pfrA*-regulated genes include the internalin operon, *inlAB* (Dramsi et al. 1993), *inlC* (Engelbrecht et al. 1996) and genes encoding several surface proteins (Sokolovic et al. 1993).

Expression of the *prfA* regulon is temperature- and growth-phase-dependent and is activated in minimal essential medium (Mengaud et al. 1991; Leimeister-Wachter et al. 1992; Sokolovic et al. 1993). Higher levels of expression of PrfA-regulated genes are observed at 37°C (compared with 25°C, as expected for an opportunistic pathogen whose virulence genes do not need to be expressed in the normal saprophytic environment; Leimeister-Wachter et al. 1992). The PrfA protein has sequence similarity to the Crp/Fnr family of transcriptional activators (Lampidis et al. 1994). It contains a helix–turn–helix motif, which is commonly found in DNA binding proteins, such as the cyclic adenosine monophosphate receptor protein, Cap or Crp, and which has been shown to be functionally significant in PrfA-mediated virulence-gene activation (Sheehan et al. 1996). PrfA binds to 14-bp regions of dyad symmetry, variants of which are found in the promoter regions of all of its target genes (Freitag et al. 1993; Dickneite et al. 1998). Differences in the regulation of the various PrfA-induced genes probably reflect differences in the PrfA-binding sites, which are located at a position 40–41 bases upstream of the transcriptional start site (Freitag et al. 1993; Bohne et al. 1994; Dickneite et al. 1998). Binding of PrfA to this PrfA box occurs independently of other factors but is more efficient in the presence of Paf (PfrA-activating factor; Böckmann et al. 1996; Dickneite et al. 1998). Enhanced Paf synthesis or activity occurs in low-iron medium. Note that, independent of any effects on the transcription of *prfA*, expression of the *hly* and *plcA* genes is repressed in the presence of cellobiose, a plant-cellulose-derived disaccharide (Park and Kroll 1993; Klarsfeld et al. 1994; Renzoni et al. 1997). It has been postulated that repression occurs because the presence of cellobiose signifies that the bacterial cells are in a saprophytic environment and do not need to produce listeriolysin O or PI-PLC (Park and Kroll 1993).

F. Role in Disease

I. Gas Gangrene

Definitive evidence for the essential role of *C. perfringens* α-toxin in clostridial myonecrosis or gas gangrene has come from independent studies in each of our laboratories (Williamson and Titball 1993; Awad et al. 1995). Immunization of mice with a recombinant protein which consisted of the C-

terminal portion (amino acids 247–370) of the α-toxin protected mice against at least ten LD_{100} doses of *C. perfringens* cells (Williamson and Titball 1993). Although this truncated protein was non-functional, antibodies prepared against it neutralised both the PLC and haemolytic activity of α-toxin. Immunoprotection was dependent upon the C-terminal component of the α-toxin, since immunization against an N-terminal derivative did not protect against the disease. Definitive genetic proof of the essential role of α-toxin in gas gangrene has come from studies that involved the construction of an insertionally inactivated chromosomal *plc* gene (Awad et al. 1995). Virulence studies carried out in mice showed that isogenic mutants that were unable to produce α-toxin were avirulent; virulence could be restored by providing a wild-type copy of the *plc* gene on a multicopy plasmid. Mice infected with the α-toxin-deficient mutants had minimal swelling, muscle destruction, inflammation or necrosis; by contrast, infection with the wild-type or complemented strains led to extensive muscle destruction and severe necrosis. Other workers have also constructed a chromosomal *plc* mutant and shown that it had significantly reduced virulence in mice (Ninomiya et al. 1994).

More recent studies have shown that α-toxin is an important modulator of neutrophil migration into infected muscle tissue (Stevens et al. 1997). One of the characteristic and unusual features of *C. perfringens*-mediated gas gangrene is the almost complete absence of polymorphonuclear leucocytes (PMNLs) in the infected lesion. Comparative histological examination of tissues derived from mice infected with either the wild-type or *plc*-mutant strains has shown that α-toxin prevents the efflux of PMNLs from the blood vessels into the muscle tissue, probably by stimulating the production of specific cell-surface adhesins in the endothelial cells. The net effect is to promote the adherence of PMNLs to the vascular endothelium, thereby facilitating the spread of the infection by preventing the influx of phagocytic cells into the infected tissues. These conclusions are supported by studies which show that purified α-toxin positively regulates the expression of the adhesins endothelial leucocyte adehesion molecule 1 and intercellular adhesion molecule 1 on the surface of endothelial cells (Bryant and Stevens 1996). Studies on cultured human endothelial cells have also revealed enhanced Paf and prostacyclin synthesis and P-selectin-mediated neutrophil adhesion in response to treatment with purified α-toxin (Bunting et al. 1997). The *C. perfringens* θ-toxin also appears to play a role in preventing PMNL influx into the lesion, perhaps in synergy with the α-toxin (Awad et al. 1995; Stevens et al. 1997).

The precise molecular pathways by which α-toxin mediates both its toxic effects and its effects on the migration of phagocytic cells are not known. However, as already discussed, they are likely to involve the stimulation of target cell regulatory cascades by the products of phospholipid hydrolysis, such as diacylglycerol. Other studies have shown that Chinese-hamster cell lines with a deficiency in their ability to produce uridine diphosphate–glucose pyrophosphorylase are hypersensitive to the cytotoxic effects of α-toxin although, again, the precise mechanism is not known (Flores-Diaz et al. 1997,

1998). Finally, studies using purified toxins in rabbits have shown that α-toxin causes systemic haemodynamic effects, probably by direct myocardial toxicity, and stimulates endogenous mediators of systemic shock, such as tumour necrosis factor (STEVENS and BRYANT 1997).

II. *P. aeruginosa* Infections

P. aeruginosa is the aetiological agent of a variety of opportunistic infections of man and is frequently isolated from the lungs of cystic-fibrosis patients and from burn tissues. The bacterium is also a pathogen of animals, causing diseases such as fleece rot in sheep, and is a pathogen of plants, causing soft rot disease. The bacterium produces a wide variety of exotoxins, including the PLC enzymes PLC-H and PLC-N (OSTROFF et al. 1990). These phospholipases show differences in substrate specificity, and only PLC-H is haemolytic. Therefore, considerable attention has been focused on PLC-H as the phospholipase most likely to play a key role in disease. Antibodies against PLC-H have been detected in sera from cystic-fibrosis patients (GRANSTRÖM et al. 1984) and from sheep suffering from fleece rot (CHIN and WATTS 1988), indicating that this enzyme is produced in vivo.

The precise roles of the *P. aeruginosa* phospholipases in the pathogenesis of disease have not been determined. The LD_{50} of PLC-H (*plcH*) mutants grown under phosphate-limited conditions in a mouse burn model is 2.4 logs greater than that of the wild-type strain (OSTROFF et al. 1989). However, this result is difficult to interpret, because mutation of the *plcR* regulatory gene, which is located downstream of *plcH*, was equally attenuating, even though the bacteria produced higher levels of PLC-H. Therefore, it is possible that the attenuation seen with the *plcH* mutant was due to a polar effect on *plcR*. Similarly, the finding that *plcH* mutants caused less severe disease in *Arabidopsis thaliana* plants (RAHME et al. 1995) might be due to polar effects on *plcR*.

Notwithstanding these results, there are several lines of evidence that suggest that the *P. aeruginosa* phospholipases do play a role in disease. Purified PLC-H administered intravenously or intraperitoneally caused necrosis of liver and kidney tissues, similar to the effects caused by crude culture filtrates, a finding which prompted the suggestion that PLC-H was the main exotoxin produced by the bacterium (MEYERS et al. 1992). PLC-H administered intradermally into sheep has been shown to cause dermal necrosis, with the infiltration of leukocytes; this resembled the lesions seen in cases of fleece rot (CHIN and WATTS 1988). Pro-inflammatory compounds, such as leukotriene B4 and histamine, could be responsible for some of these effects, and these compounds are released from human granulocytes treated with PLC-H (KÖNIG et al. 1996). The effects were further potentiated by the lipase produced by *P. aeruginosa*, suggesting that these enzymes might act synergistically in vivo (KÖNIG et al. 1996).

What role might the PLCs play in disease? One possibility is that the phospholipases form part of a phosphate-retrieval system, which might be impor-

tant because the level of free phosphate in sera is sub-optimal for the growth of Gram-negative pathogens (Weinberg 1974). The finding that PLC production is induced under low-phosphate conditions (Pritchard and Vasil 1986; Ostroff et al. 1990; Shortridge et al. 1992) supports this suggestion. In respiratory-tract infections, the enzymes might play additional roles by degrading lung surfactant (Holm et al. 1991), which is rich in PC, to allow the bacterium to gain access to underlying tissues. Another intriguing possibility is that the phospholipases may play a role in the accumulation of choline, which then accumulates within the bacterial cell and acts as an osmoprotectant in the lung (Landfeld and Strom 1986; Sage et al. 1997).

III. The Pathogenesis of Listeriosis

L. monocytogenes is a food-borne pathogen that causes severe meningitis and septicaemia, particularly in elderly or immunocompromised patients, the new born and in pregnant women, where it may cause abortion. Pathogenesis is primarily dependent on the ability of the organism to survive and replicate in macrophages (Sheehan et al. 1994). *L. monocytogenes* is an invasive intracellular pathogen that avoids the host immune system by transferring directly from one cell to the adjoining cell. This process involves the ActA-mediated movement of listerial cells within and between the mammalian host cells by a process of actin polymerisation (Tilney and Portnoy 1989; Sheehan et al. 1994). After initial entry of *L. monocytogenes* into the macrophage by phagocytosis, the organism escapes the resultant primary phagocytic vacuole, mainly because of its ability to produce the pore-forming cytolysin, listeriolysin O. PI-PLC also plays a role in the escape of the cells from phagosomes of the macrophage, enabling the organism to grow in the cytoplasm of the host cell (Camilli et al. 1993). ActA enables the bacteria to move through the host cells and into neighbouring cells, forming a membrane-bound protrusion. PC-PLC is believed to be important in cell-to-cell spread, probably by hydrolysing phospholipids in the cell membrane and enabling the bacteria to escape these membrane-bound protrusions (Vazquez-Boland et al. 1992). A mutant that was unable to produce PC-PLC has been shown to be defective in cell-to-cell spread (but not in escape from the primary phagocytic vacuole) and was 20 times less virulent in mice (Smith et al. 1995). An equivalent PI-PLC mutant had only a twofold reduction in virulence and was not affected in its ability to spread directly from one cell to another, although delayed escape from the primary vacuole was observed. Double *plc* mutants unable to produce either enzyme are more than 500 times less virulent than the isogenic wild-type strain and are severely affected in both intracellular spread and vacuolar escape (Smith et al. 1995).

Recent virulence studies have also shown that PC-PLC is important in cerebral listeriosis in the mouse; when mice are infected with a *plcB* mutant, death is significantly delayed compared with that in wild-type mice (Schlüter et al. 1998). In summary, both phospholipases are important in virulence, with

PI-PLC required for efficient escape from the primary vacuole and PC-PLC required for cell-to-cell spread. In epithelial cells, PC-PLC also appears to be involved in escape from the phagosome, at least in the absence of listeriolysin O, since mutants lacking listeriolysin O and PC-PLC are unable to grow intracellularly, unlike mutants lacking listeriolysin O and PI-PLC (MARQUIS et al. 1995). Neither phospholipase appears to be involved on its own in the stimulation of neutrophil adhesion to endothelial cells, a process that is presumably important in the systemic spread of *L. monocytogenes* cells (DREVETS 1998). However, even in the absence of cell invasion, PI-PLC and listeriolysin O act synergistically to induce endothelial cell signalling and inflammatory events (SIBELIUS et al. 1996). It is proposed that pore formation by listeriolysin O is required for the uptake of PI-PLC and its subsequent stimulation of signal-transduction pathways in the endothelial cell.

IV. Caseous Lymphadenitis in Ruminants

Caseous lymphadenitis is a disease of the lymphoid tissues of ruminants, particularly sheep and goats, and is caused by *C. pseudotuberculosis*. Virulent biovars of *C. pseudotuberculosis* produce a PLD that has been shown to be essential for virulence in sheep and goats (HODGSON et al. 1992; MCNAMARA et al. 1994). The organism appears to be an intracellular pathogen that multiplies in the phagolysosomes of neutrophils and macrophages and produces abscesses, primarily at the site of infection and in the regional lymph nodes. Infection of goats with a *C. pseudotuberculosis* strain carrying a chromosomal *pld* mutation led to a markedly reduced disease pathology, with minimal dissemination of the organism from the primary site of infection and little evidence of abscessation (MCNAMARA et al. 1994). Using a different chromosomal *pld* mutant, similar results have been obtained in sheep (HODGSON et al. 1992). Despite numerous studies on the use of rationally attenuated *C. pseudotuberculosis* strains as vaccines (HODGSON et al. 1992; SIMMONS et al. 1997, 1998), very little is known about the role of PLD in the disease process.

G. Conclusions

Work with bacterial PLCs during the 1940s founded many principles that were then subsequently applied to the study of other bacterial toxins. Perhaps because of these early studies, interest in bacterial phospholipases waned during subsequent decades, and it was not until the 1990s that there was a resurgence of interest in this group of bacterial toxins. Today, a wide variety of pathogenic bacteria are known to produce phospholipases and, from the early findings that some of these enzymes were toxins, we have developed an appreciation of the diversity of roles that these enzymes play in the pathogenesis of disease. In spite of this knowledge, there are many questions yet to be answered. Do these enzymes play a role in the colonisation of phospho-

lipid-rich mucosal surfaces? Why do some pathogens produce several enzymes with different properties? What roles do these enzymes play in the role of diseases caused by *M. tuberculosis*, *B. pseudomallei* and *Helicobacter pylori*? At a molecular level, we know little about the mechanisms of action of these enzymes. How do these enzymes interact with membrane phospholipids, and what is the molecular basis of the subtle but important differences in substrate specificity? Do phospholipases produced by intracellular pathogens play a role in modulating cell metabolism to the advantage of the pathogen? How is the expression of bacterial phospholipases regulated in the infected tissues?

Many of the techniques and tools required to answer these questions are now available and are being applied to the study of this group of enzymes. These studies are of more then academic interest. Enzymes that play key roles in the pathogenesis of disease might be modified to provide vaccines, and inhibitors of these enzymes might find places as new anti-bacterial drugs to deal with antibiotic-resistant strains of bacterial pathogens. These enzymes might also find applications in novel drug-delivery systems, such as that described by CARTER et al. (1998). This is a truly exciting time to be working in this field.

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CHAPTER 24

Pore-Forming Toxins as Cell-Biological and Pharmacological Tools

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A. Permeabilized Cells: an Approach to the Study of Intracellular Processes

Using permeabilized cells, intracellular processes can be studied under conditions that are believed to resemble closely the physiological ones in intact cells. In the past, various experimental approaches have been developed, including electropermeabilization (KNIGHT and BAKER 1982; BARTELS et al. 1994), application of digitonin (BITTNER et al. 1986), mechanical shearing of cells by a cell cracker (MARTIN 1989) and use of bacterial pore-forming toxins. Actually, electropermeabilization was the first approach to gain access to the cell interior and resulted in a number of data concerning the minimal requirements for exocytosis in chromaffin cells (KNIGHT and BAKER 1982). Recent investigations used this method in studies of signal transduction events (for example, in adipocytes; KOKKALIARI et al. 1994) and intracellular Ca^{2+} mobilization in neuroblastoma cells (WOOD et al. 1994). However, electropermeabilization is often transient, and the cells may repair the holes, which limits its application during an experiment. The transient stage of the holes may, however, be an advantage in introducing low- and high-molecular-weight substances into cells, where they persist for long times. This approach has been elegantly used for the introduction of clostridial neurotoxins into chromaffin cells, allowing long-term incubations and giving information on how these toxins are handled by the cells (BARTELS and BIGALKE 1992; BARTELS et al. 1994; BINSCHECK et al. 1995; ERDAL et al. 1995). For short-term permeabilization, which allows immediate dissection of intracellular events, electropermeabilization can be used mainly for cells in suspension and is difficult to apply to a great number of probes with attached cells.

Using PC12 cells, cell cracking has led to many exciting data concerning the events during exocytosis, where a priming step and a fusion step could be distinguished (HAY and MARTIN 1993; HAY et al. 1995), and on the biogenesis of secretory granules (TOOZE et al. 1991; DITTIE et al. 1996; 1997). Similar to electropermeabilization, cell cracking requires cells in suspension at a high density and cannot be applied to attached cells.

Permeabilization by bacterial pore-forming toxins like α-toxin from *Staphylococcus aureus* (strain Wood 46) and streptolysin O (SLO) from group A β-hemolytic streptococci is now a widely accepted approach in the func-

tional analysis of intracellular organelles. In contrast to the methods of cell cracking and electropermeabilization, pore-forming toxin can be applied to various preparations, including cells in suspension, attached cells, neurons and synaptosomes (DEKKER et al. 1989; STECHER et al. 1992; BOBICH and ZHENG 1995; AHNERT-HILGER and WELLER 1997).

Storage and release of neurotransmitters by secretory vesicles are our main interest. For example, the regulation of exocytotic membrane-fusion processes involves the concerted interaction of two membranes: that of the secretory vesicle and the plasma membrane. Permeabilized cells are ideally suited for these studies, since they allow the manipulation of the intracellular environment without affecting membrane function. In recent years, permeabilized preparations have been used to study regulated (i.e., Ca^{2+}-dependent) membrane fusion from a great variety of secretory cells, including the rat pheochromocytoma cell line PC 12 (AHNERT-HILGER et al. 1985), bovine chromaffin cells in primary culture, mast cells, cytotoxic T-lymphocytes, gonadotropic cells of the adenohypophysis, insulin- secreting cells (LANG et al. 1995, 1997), isolated exocrine pancreatic cells (STECHER et al. 1992) and synaptosomes (BHAKDI et al. 1993; AHNERT-HILGER and WELLER 1997). In addition to regulated exocytosis, which depends on an increase in the intracellular Ca^{2+} concentration, other regulated fusion processes were successfully studied using either SLO- or α-toxin-permeabilized cells. Regulations underlying the translocation of the glucose transporter in adipocytes and cardiomyocytes were successfully analyzed after permeabilization by either pore-forming toxin. In addition, the analysis of intracellular Ca^{2+} regulation (FÖHR et al. 1991), glucose and arachidonic acid metabolism, stimulus–contraction coupling (KITATAWA et al. 1989), intracellular membrane trafficking and Ca^{2+}-regulated fusion of lysosomes in fibroblasts and epithelial cells (RODRIGUEZ et al. 1997) were also addressed by this approach (BHAKDI et al. 1993; AHNERT-HILGER and WELLER 1997 and references cited therein). Permeabilized preparations are also suitable for study of Golgi-membrane dynamics (JAMORA et al. 1997; MIRONOV et al. 1997). Very recently, the regulation of vesicular transporters, especially the monoamine transporter, by heterotrimeric G-proteins (AHNERT-HILGER et al. 1998) was analyzed in permeabilized preparations.

The native forms of the toxins assemble into amphiphilic polymers in the target lipid bilayers, where they generate stable transmembrane pores. α-Toxin pores comprise a homogenous population of ring-structured heptamers (GOUAUX et al. 1994) that allow for the free passage of low-molecular-mass solutes (BHAKDI and TRANUM-JENSEN 1987; BHAKDI et al. 1993). SLO pores are heterogeneous, with larger diameters, which permits the exchange of proteins (BHAKDI and TRANUM-JENSEN 1987; AHNERT-HILGER et al. 1989b; BHAKDI et al. 1993).

B. α-Toxin and SLO as Tools with which to Study Functional Aspects of Intracellular Organelles

The characteristic features of both toxins can be used to address various problems concerning the regulation of intracellular organelles. α-Toxin permeabilizes cells for small molecules (≤3 kDa). α-Toxin monomers bind to as yet unidentified cell-surface acceptor sites that are present (in varying amounts) on most nucleated cells. When applied at high concentrations (50–200 μg/ml), a non-specific adsorption of lipids in almost any cell type occurs, resulting in pore formation and permeability to small molecules (Bhakdi et al. 1993). SLO monomers bind to cholesterol. After oligomerization and pore-formation, cells are permeable to both small and large (≥150 kDa) molecules. In this respect, SLO resembles digitonin, whose membrane-permeabilizing effects are, however, more difficult to handle (Ahnert-Hilger et al. 1989b; Bhakdi et al. 1993; Ahnert-Hilger and Weller 1997).

I. Biological Activity and Cell Permeability

α-Toxin from *S. aureus* (strain Wood 46, ATCC 10832, DSM 20491) can be purified easily from the culture supernatant (Lind et al. 1987; Palmer et al. 1993). The purified toxin can be lyophylized or, if necessary, dialyzed against an intracellular buffer (see below). Dissolved α-toxin can be stored in aliquots at –20°C for many months without loss of activity.

SLO is purified from the culture supernatant of group-A β-hemolytic streptococci (*S. pyogenes*; Pinkney et al. 1995). The excellent final product (kindly provided by U. Weller, Institute Rai, Roecky, Weller, Baden-Baden, Germany) can be stored in aliquots at –20°C for many years. SLO is only active in the reduced form; thus, addition of 1 mM dithiothreitol (DTT) is recommended (Pinkney et al. 1995; Weller et al. 1996; Ahnert-Hilger and Weller 1997).

Native SLO and α-toxin are commercially available. Crude α-toxin preparations with low titer can be concentrated by fractionated ammonium-sulfate precipitations (first precipitation with 55%, and supernatant subjected to a second precipitation with 65%) followed by dialysis against any suitable buffer (Schrezenmeier et al. 1988). The biological activity of some SLO preparations (Institut Pasteur, Paris, France; Wellcome Diagnostics, Dartford, UK) is given in international units. These apply when determining the anti-SLO titer and cannot be compared directly with the hemolytic units (HU) mentioned below. These materials, however, represent freeze-dried preparations of partially purified culture filtrates contaminated with large amounts of reducing cysteine and, when used without further purification, may affect the processes under study. For other commercially available products, see Sect. C.

1. Protocol 1: Permeabilization of Attached Cells by α-Toxin or SLO

1. Cultivate cells on an appropriate plastic dish, i.e., a 24-multi-well dish. Cell density may be variable and depends on cell type and the problems under study. Best results will probably be obtained with semiconfluent cells. However, the amount of α-toxin or SLO has to be increased when increasing the number of cells.
2. Wash cells with an extracellular buffer, i.e., phosphate-buffered saline (PBS; see protocol 3.2) or Krebs' buffer (see protocol 7.7).
3. Wash cells with an intracellular buffer consisting mainly of potassium, i.e., KG III buffer (see protocol 7.5).
4. Incubate cells in intracellular buffer with α-toxin for 10–30 min at 25–37°C or SLO for 2–10 min at 4°C.
5. Remove toxin solution.
6. Incubate cells with substances to be tested dissolved in an intracellular buffer at 25–37°C, depending on the experimental conditions.

a) Alternate Protocol 1

1. As above
2. As above
3. As above
4. Incubate for 10 min at 30°C or 37°C with 20 μM digitonin dissolved in intracellular buffer
5. As above
6. As above

2. Protocol 2: Permeabilization of Cells in Suspension

1. Cells cultivated either attached or in suspension may be used. For some protocols, i.e., secretion studies, cells may be preloaded with radioactive neurotransmitter before being detached and permeabilized. The decision to use cells in suspension depends on whether attached cells will detach during permeabilization or when using Ca^{2+}-free buffers.
2. Wash cells with an extracellular buffer, i.e., PBS (see protocol 3.2) or Krebs' buffer (see protocol 7.7). This step can be performed before detaching.
3. Wash cells with an intracellular buffer consisting mainly of potassium, i.e., KG buffer. This step can be performed before detaching.
4. Detach cells by gently pipetting. Try to get a single cell suspension. This will decrease variations in your results. The use of trypsin may be required.
5. Incubate cells in intracellular buffer with α-toxin for 10–30 min at 25–37°C or SLO for 2–10 min at 4°C. This step can be performed in batch or after having distributed cells to individual samples.
6. Remove the toxin solution by low-speed centrifugation 1–2 min at 4°C and 2000–5000 × *g*.

7. Incubate cells with substances to be tested at 25–37°C, depending on the experimental conditions. Add test solution to each sample and gently re-suspend by pipetting. If permeabilization has been performed in batch, distribute cells to individual samples and then add test solution.

a) Commentary for Protocols 1 and 2

The protocols given above have been worked out for PC12 cells either attached to culture plates or re-suspended. For primary cultures of bovine chromaffin cells, only protocol 1 has been performed (AHNERT-HILGER et al. 1989). Protocol 2 also works for freshly prepared pancreatic acinar cells (STECHER et al. 1992). The protocols may be applied to any other cell type. However, concentrations of all three permeabilizing agents have to be determined individually for each cell type. Depending on the function under study, permeabilized cell preparations have to be used in minutes or can be kept on ice for hours (FÖHR et al. 1991; AHNERT-HILGER and WELLER 1997). For intermediate incubations, a representative experiment will last between 2 h and 4 h.

In contrast to digitonin, a general advantage of both pore-forming toxins is that their damaging effects can be restricted to the plasma membrane. In the case of α-toxin, this is due to the fact that the α-toxin monomers are too large (34 kDa) to pass the pores generated by the heptamer (BHAKDI and TRANUM-JENSEN 1987; BHAKDI et al. 1993). Accordingly, proteins are not lost during permeabilization. The large pores generated by the SLO monomers after oligomerization also let proteins pass. To avoid damage of intracellular membranes by SLO monomers, the cells are incubated for a very short period of time or at 0°C. Under the latter conditions, the SLO monomers just bind to the plasma membrane. After washing, pore formation is initiated by warming the preparation. In contrast to SLO, membrane permeabilization with digitonin is insensitive to temperature and, therefore, is more difficult to control (AHNERT-HILGER et al. 1989b).

The biological activity of various preparations of both toxins can be checked easily using rabbit red blood cells. Usually, a red blood cell suspension (2.5% final concentration) in PBS (see protocol 3.2) supplemented with 4% sodium citrate is mixed with various toxin dilutions (final volume 55 μl). Samples are incubated at 37°C for 40 min, after which hemolysis is monitored spectrophotometrically at 412 nm using 30 μl of the supernatant diluted with 1 ml of distilled water. Total hemolysis is determined after the addition of sodium dodecylsulfate (SDS, 0.2%), which gives an extinction of about 1.2.

3. Protocol 3: Assay to Compare Biological Activity of Various Pore-Forming Toxins Using Rabbit Erythrocytes

Solutions

1. 4% sodium citrate. To make 100 ml, solubilize 4 g of sodium citrate in distilled water and adjust to a total volume of 100 ml.

2. PBS. To make 1 l, dissolve 6.9 g $NaH_2PO_4 \cdot 2H_2O$ and 7.6 g NaCl in about 600 ml of distilled water, titrate to pH 7.2 with NaOH and adjust to a total volume of 1 l. Store at 4°C.
3. 2% SDS. To make 100 ml, dissolve 2 g SDS in 100 ml of distilled water.

Steps

1. Mix nine volumes of fresh rabbit blood with one volume of 4% sodium citrate
2. Wash three times with PBS by a 5-min centrifugation at $3000 \times g$
3. Dilute the erythrocyte pellet 1:40 in PBS, which gives an approximately 2.5% erythrocyte suspension
4. Mix 5 µl of toxin (diluted in PBS, in the case of SLO in PBS supplemented with 1 mM DTT) either with 5 µl PBS alone for control or with 5 µl 2% SDS with 50 µl of the 2.5% erythrocyte suspension
5. Incubate, with constant shaking, in a water bath for 40 min at 37°C
6. Centrifuge for 2 min at $12000 \times g$
7. Remove 30 µl of the supernatant and dilute with 1 ml of water
8. Estimate the hemoglobin content spectrophotometrically at 412 nm

a) Commentary for Protocol 3

The reciprocal dilution of the toxin hemolyzing 50% of the erythrocyte suspension at 37°C within 40 min is taken as the number of hemolytic units per milliliter of the undiluted toxin solution (LIND et al. 1987; AHNERT-HILGER et al. 1989b). The red blood cell test allows comparison of various toxin preparations of α-toxin or SLO. Rabbit erythrocytes are the most sensitive ones. The test takes about 2 h, starting with bleeding the animal. A rabbit red cell suspension can be stored in PBS for a few days at 4°C.

Individual permeability of a given cell population can be easily checked with membrane-impermeable dyes that stain either various components of the cell body (trypan blue or eosin) or only the nucleus (azur A). Toxin-treated cells are dyed in an isotonic medium containing a final dye concentration of 0.2%.

4. Protocol 4: Trypan-Blue Exclusion Test

Solutions

1. PBS (see protocol 3)
2. Trypan blue

Steps

1. Suspend about $3–5 \times 10^5$ cells in 50 µl PBS (see hemolytic assay support protocol 1) and add 5 µl of toxin dilution containing between 10 HU and 1000 HU.
2. Incubate the mixture for 5 min at 36°C, then store on ice.
3. Mix 10 µl of this suspension with 10 µl of trypan blue solution on a glass slide and immediately check the staining of the cells under a light microscope.

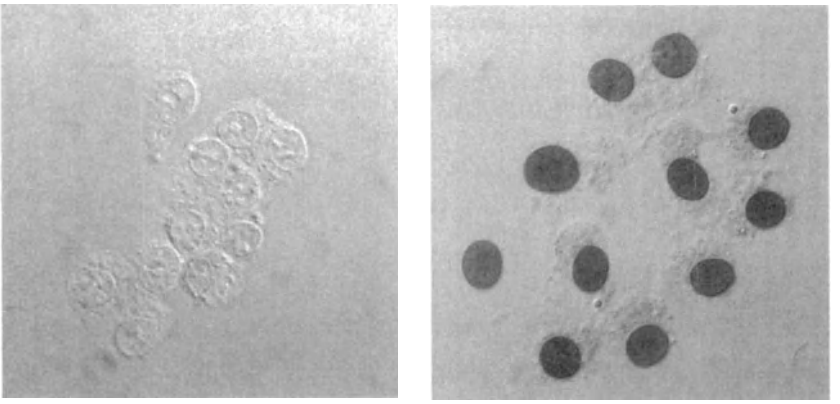

Fig. 1. Trypan-blue exclusion test in PC12 cells. A complete staining of the cells with trypan blue is only observed in streptolysin-O-treated cells (*right panel*) but not in control cells (*left panel*). *Bar* 10 μm.

4. For control, mix 50 μl of cell suspension with 5 μl of PBS. The control cell suspension should exclude the dye. Use the toxin dilution that leads to a staining of about 90–95% of the treated cells (Fig. 1).

a) Commentary for Protocol 4

The percentage of stained cells should immediately be determined under the light microscope, since prolonged incubation may lead to staining of intact cells. Fig. 1 gives an example of the trypan-blue exclusion test using the rat pheochromocytoma cell line PC12 after applying 100 HU of SLO to the cells. The trypan-blue test is the easiest way to rapidly check the biological activity of a new batch of toxin or the conditions for permeabilization of a certain cell type. It is the first step when trying to address a special experimental question about permeabilized cells (Ahnert-Hilger et al. 1985). It may also be used to check permeability during an experiment. It uses only minimal amounts of cells and takes about 1 h or less. The test, however, does not allow one to distinguish whether the process under study is still functioning.

II. Introduction of Membrane-Impermeable Proteins

Permeabilization with SLO allows the introduction of antibodies directed against intracellular proteins (Ahnert-Hilger et al. 1989b). The principle is outlined in Fig. 2.

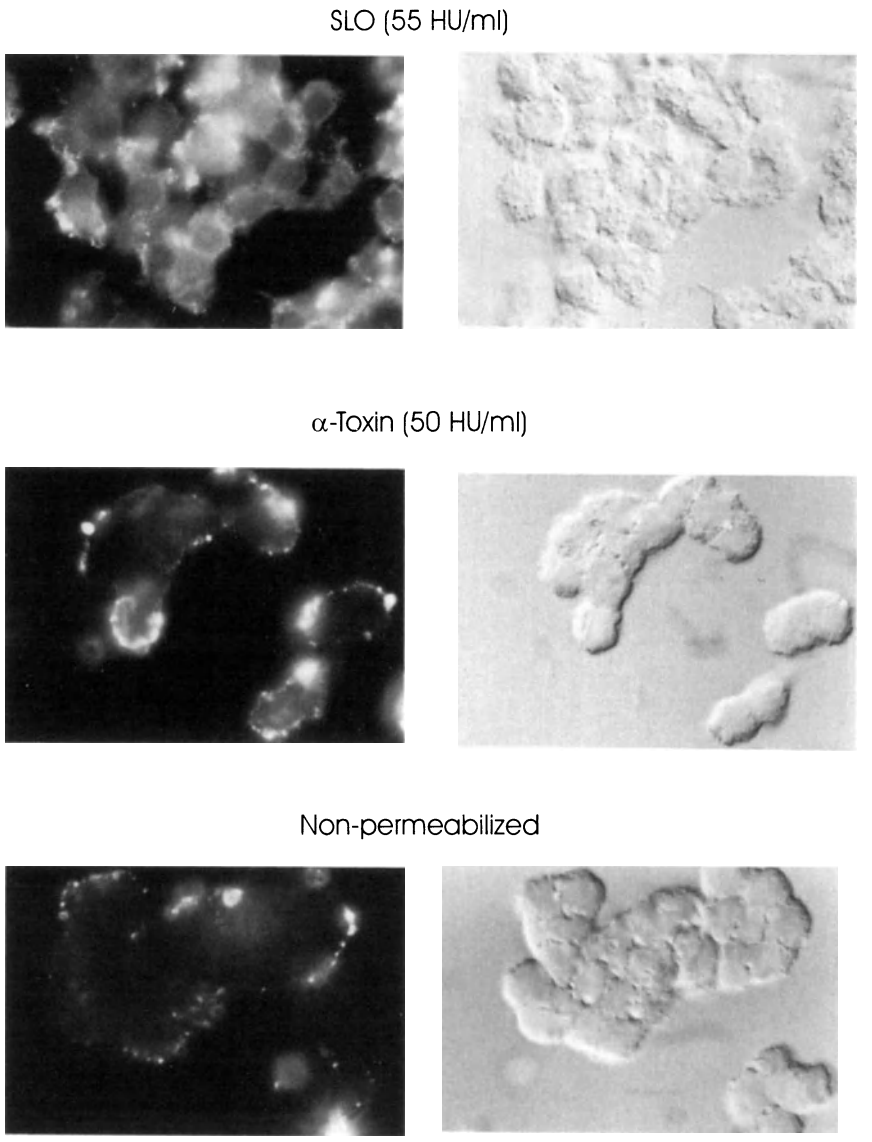

Fig. 2. Immunofluorescence microscopic detection of synaptophysin in streptolysin O (SLO)- or α-toxin-permeabilized PC 12 cells. The polyclonal antiserum against synaptophysin only gains access to the intracellular vesicle protein when cells have been permeabilized with SLO (*upper panels*). In α-toxin-permeabilized (*middle panels*) or non-permeabilized (*lower panels*) cells, the antiserum stains only small patches on the cell surface, presumably due to sites of spontaneous exocytotic events. The *left panels* show the immunofluorescence, the *right panels* the respective phase contrast. *Bar* 10 μm. *HU*, hemolytic units.

1. Protocol 5: Introduction of Membrane-Impermeable Proteins. Immunofluorescence for Synaptophysin

Solutions

1. 4% formalin. Solution A: 0.2 M $NaH_2PO_4 \cdot 2H_2O$ (31.2 g/l H_2O). Solution B: 0.2 M Na_2HPO_4. Mix 19 ml A and 81 ml B. Adjust to 200 ml with H_2O. Weigh 8 g paraformaldehyde and dissolve in 100 ml at 60°C. Add 10 N NaOH and stir until a clear solution is obtained. Cool down. Adjust pH to 7.4 by adding HCl and add 100 ml of the 0.2 M phosphate buffer. Store in aliquots at –20°C.
2. Bovine serum albumin (BSA) fraction V, pH 7.0, Serva.
3. Normal goat serum (NGS), PAN Systems GmbH.
4. Goat-anti-antiserum Cy3-labeled, Jackson Dianova.

Steps

1. Cultivate cells on glass cover slides.
2. Rinse once with PBS.
3. Incubate cells with either α-toxin or SLO dissolved in PBS supplemented with 1 mM DTT. Permeabilization by α-toxin is achieved by incubating the cells for 30 min at 37°C, whereas SLO treatment is performed for 10 min on ice.
4. Rinse twice with PBS.
5. Incubate for 10 min at room temperature with PBS supplemented with 5% NGS and 2% BSA. Under these conditions, the bound SLO monomers polymerize and form stable transmembrane pores.
6. Incubate cells for 2 h at 37°C with antibodies diluted in PBS/NGS/BSA. Here, a polyclonal antiserum against synaptophysin (kindly provided by Dr. R. JAHN, MPI für biophysikalische Chemie, Göttingen, Germany) was used.
7. Remove the primary antibody by rinsing the cells three times with PBS.
8. Fix cells in 4% formalin for 10 min at 4°C.
9. Rinse fixed cells five times with PBS and incubate them with a secondary antibody (here an anti-rabbit antiserum coupled to Cy3 was used) dissolved in PBS containing 2% BSA for 2 h at room temperature.
10. Remove the solution and rinse cells three times with PBS.
11. Mount coverslips on glass slides and analyze the fluorescence signal with a fluorescence microscope.

a) Commentary for Protocol 5

The whole experiment takes about 6 h. Incubation with the secondary antibody after fixation can also be performed overnight. As expected, the antiserum against synaptophysin gives a broad intracellular fluorescence only after permeabilization with SLO. Digitonin can also be used for this purpose and, for some cell-types, may be even superior to SLO. Due to the pore size generated by α-toxin, the antibodies do not get access to intracellular antigens

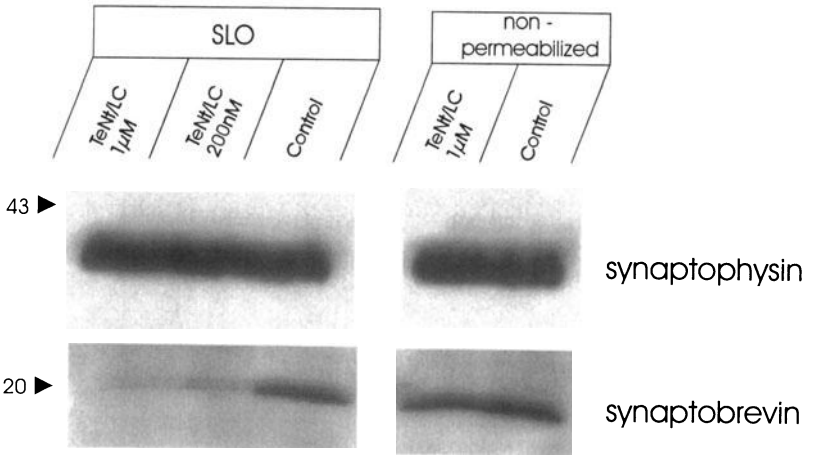

Fig. 3. Cleavage of synaptobrevin by tetanus-toxin light chain (TeNt/LC) in streptolysin-*O*-permeabilized PC12 cells. The position of molecular-weight markers in kiloDaltons is given on the left. Cleavage of synaptobrevin by TeNt/LC is achieved only in the permeabilized cells. Non-permeabilized cells (*right lanes*) cannot take up TeNt/LC; therefore, synaptobrevin remains uncleaved. Note that the synaptophysin content did not change under either condition.

(Ahnert-Hilger et al. 1989). This protocol may be applied to any other cell type, using antibodies to various kinds of intracellular antigens.

SLO-permeabilized cells may also be used when one wants to knock out intracellular proteins biochemically. This approach was first used to study the intracellular effects of clostridial neurotoxins, e.g., tetanus toxin (TeNt), in secretory cells which, if untreated, are insensitive to the holotoxins. Using various permeabilized neuroendocrine cells, the biological effects of the TeNt heavy and light chains could be studied separately. Once inside the cell, the light chain of TeNt (TeNt/LC) proteolytically cleaves the vesicular protein synaptobrevin (Ahnert-Hilger and Bigalke 1995). Cleavage of synaptobrevin in PC12 cells by TeNt/LC (Fig. 3) is described here as an example of the introduction of biologically active proteins into permeabilized cells.

2. Protocol 6: Introduction of Membrane-Impermeable Proteins. TeNT/LC

Solutions

1. KG buffer III (see protocol 7).

Steps

1. Suspend PC12 cells (about 5×10^5) in 25 μl KG buffer (Sect. B.III) in an Eppendorf cup and mix with 25 μl SLO (diluted to 200 HU/ml in KG buffer).
2. Incubate the mixture for 10 min at 4°C followed by centrifugation for 1 min at $2000 \times g$.

3. Remove the supernatant and re-suspend the cell pellet in KG buffer supplemented with either 1 μM or 200 nM TeNt/LC. SLO-treated controls receive only KG buffer. Non-permeabilized cells are subjected to the same procedure, receiving either no toxin or 1 μM TeNt/LC.
4. Incubate samples at 36°C for 20 min. After centrifugation at 14000 $\times$ *g* for 1 min, the supernatant is removed and the pellet dissolved in 30 μl 0.2% SDS (protocol 7).
5. Use about 5 μl to determine protein content by the bicinchoninic-acid (BCA) method. Dilute the rest 3:1 with Laemmli buffer and subject to SDS polyacrylamide-gel electrophoresis followed by transfer to nitrocellulose.
6. Cut nitrocellulose into strips incubated with antibodies against synaptobrevin (18 kDa) or synaptophysin (38 kDa). Analyze by standard techniques (enhanced chemiluminescence or alkaline phosphatase).

a) Commentary for Protocol 6

Since intact PC12 cells are insensitive to TeNt/LC, synaptobrevin is not cleaved. After permeabilization with SLO, TeNT/LC (200 nM or 1 μM) almost completely cleaves synaptobrevin, whereas the amount of synaptophysin tested for comparison remains unchanged. If the test does not work, check permeability with the trypan-blue exclusion test. The enzymatic activity of the clostridial neurotoxin light chain may be tested with recombinant synaptobrevin (Ahnert-Hilger and Bigalke 1995).

III. Assay for Exocytosis in Permeabilized Cells

Permeabilized secretory cells are widely used to study the final events that occur during secretion by exocytosis. Cellular models include bovine adrenal chromaffin cells in short-time culture and the rat pheochromocytoma cell line PC12. Both cell types take up labeled catecholamines and store them within secretory vesicles, from which they can be released upon stimulation. The released catecholamines can be detected in the supernatant. After permeabilization of the plasma membrane, release of catecholamines can be triggered by micromolar concentrations of Ca^{2+}.

In most of the studies dealing with exocytosis by permeabilized cells, the medium used reflects intracellular conditions, containing potassium as a main cation and glutamate as an anion. Since the free-Ca^{2+} concentration within the cell is in the micromolar range under resting conditions and during stimulation, this ion must be carefully controlled in the buffers used. A combination of chelators for divalent cations is suitable to buffer the free-Ca^{2+} concentration between 0.1 μM and 100 μM under the experimental conditions. Added Mg^{2+} and adenosine triphosphate (ATP) and the pH of the medium must be considered, because they alter the equilibrium between Ca^{2+} and the chelators present. The free Ca^{2+} and Mg^{2+} concentrations are calculated using a com-

puter program and controlled by Ca^{2+}- and Mg^{2+}-specific electrodes (FÖHR et al. 1993). Each Ca^{2+} buffer is prepared separately from stock solutions, with a final check of pH, pCa and pMg. If no Ca^{2+} electrode is available, the calculated total amount of Ca^{2+} (as $CaCl_2$) and Mg^{2+} [as $Mg(CH_3CO_2)_2$] must be added before the pH adjustment. Buffers can be stored at –20°C but should be thawed only once, mainly because of decomposition of ATP.

The experimental procedures using α-toxin or SLO to permeabilize the plasma membranes of both PC12 and adrenal chromaffin cells are given below. The secretory response decreases with time after permeabilization. The decrease varies between different cell types and preparations. In SLO-permeabilized PC12 cells, exocytosis can be restored using cytosolic fractions. The free-Ca^{2+} concentration necessary to elicit exocytosis varies between 1 μM and 10 μM, depending on the cell preparations, but is always three- to fivefold higher in α-toxin-permeabilized cells. Since the permeability to Ca^{2+} is the same irrespective of whether the cells are treated with α toxin, SLO or digitonin (FÖHR et al. 1991), buffering by intracellular proteins may be the reason for the observed differences in Ca^{2+} sensitivity.

1. Protocol 7: Measuring Exocytosis in Permeabilized Suspension Cells

Solutions (examples of KG buffers used in the analysis of exocytosis from permeabilized chromaffin or PC12 cells)

1. Prepare 1 M $Mg(CH_3COO)_2$ stock solution by dissolving 2.145 g $Mg(CH_3COO)_2 \cdot 4H_2O$ in 10 ml of distilled water.
2. Prepare 1 M $CaCl_2$ stock solution by dissolving 1.47 g $CaCl_2 \cdot 2H_2O$ in 10 ml of distilled water.
3. KG buffer I with Mg^{2+}/ATP for permeabilization and incubation before the stimulation [150 mM K^+–glutamate, 2 mM ethylene glycol tetraacetic acid (EGTA), 2 mM ethylene diamine tetraacetic acid (EDTA), 20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid; Pipes), 2 mM Na^+/ATP, 1 mM free Mg^{2+}]. For 500 ml, dissolve 13.9 g K^+–glutamate, 0.38 g EGTA (free acid), 0.2 g EDTA (free acid), 3.02 g Pipes (free acid) and 0.61 g Na^+/ATP in about 300 ml of water, add KOH to a pH of 7.0 and stir at room temperature. Add 2.4 ml of 1 M $Mg(CH_3COO)_2$, which will give a final free-Mg^{2+} concentration of 1 mM, and adjust the pH to exactly 7.0 with KOH. Adjust to 500 ml.
4. KG buffer II with Mg^{2+}/ATP and Ca^{2+} (15 μM, free) for stimulation. For 100 ml, dissolve one-fifth of the amount of the salts listed for KG buffer I in about 60 ml. Add 370 μl of 1 M $Mg(CH_3COO)_2$ and 310 μl of 1 M $CaCl_2$, which will give a final free-Mg^{2+} concentration of 1 mM and a final free-Ca^{2+} concentration of 15 μM. Adjust the pH to exactly 7.0 with KOH. Adjust to 100 ml.
5. KG buffer III without ATP and Ca^{2+}. For 500 ml, the procedure is the same as described above, except that Na^+/ATP is left out. Add 1.55 ml

of $Mg(CH_3COO)_2$, which will give a final free-Mg^{2+} concentration of 1 mM, and adjust the pH to exactly 7.0 with KOH. Adjust to 500 ml.

6. KG buffer IV without ATP but with Ca^{2+} (15 μM, free). For 100 ml, the procedure is the same as for KG buffer II except that Na^+/ATP is left out. Add 190 μl of 1 M $Mg(CH_3COO)_2$ and 310 μl of 1 M $CaCl_2$, which will give a final free-Mg^{2+} concentration of 1 mM and a final free-Ca^{2+} concentration of 15 μM. Adjust the pH to 7.0 and adjust to 100 ml.
7. Ca^{2+}-free Krebs' buffer (140 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 20 mM Pipes, 11 mM glucose, pH 7.0). For 1 l, weigh the following substances: 8.2 g NaCl, 0.35 g KCl, 0.16 g KH_2PO_4, 0.3 g $MgSO_4 \bullet 7H_2O$ and 4.8 g HEPES (free acid). Dissolve in 800 ml of distilled water, adjust the pH to 7.0 with NaOH and adjust to 1 l. Store in appropriate aliquots at –20°C. Add glucose (0.218 g per 100 ml) freshly before use.
8. 100 mM ascorbic acid. For 10 ml, weigh 0.176 g and dissolve in distilled water. Store in aliquots at –20°C.
9. I-[7,8-3H] Noradrenaline (3H-NE, 15 Ci/mmol; Cat No. TRA.584, Amersham Buchler, Braunschweig, Germany).
10. To load one 100-mm culture dish, mix 100 μl 100 mM ascorbic acid with 10 μl 3H-NE and 10 ml of Dulbecco's modified essential medium (DMEM). For one multi-plate with 24 wells, mix 66 μl of 100 mM ascorbic acid with 7 μl 3H-NE and 6.6 ml DMEM. Load each well with 250 μl.

Steps (assay for exocytosis using PC12 cells in suspension)

1. Label one 100-mm dish with 10 ml 3H-NE solution for 2 h in the cell incubator.
2. Aspirate the labeling medium and chase cells for 1–2 h in DMEM.
3. Aspirate DMEM and wash cells with Krebs' buffer four times for 5 min.
4. Wash cells once with 10 ml KG buffer I or III.
5. Suspend cells in 0.6–0.9 ml KG buffer I or III and divide into 12–18 vials (50 μl each), corresponding to about 2–3 $\times$ 10^5 cells/sample already containing 50 μl of 100 HU/ml α-toxin or SLO corresponding to about 300–500 HU/10^7 cells. Incubate for 20–30 min at 25, 30 or 37°C with α-toxin or 5 min on ice with SLO.
6. Centrifuge each sample at 3000 $\times$ g for 20 s and remove supernatant.
7. Resuspend pellet in 100 μl of KG buffer I or III containing the substances to be tested, and incubate for 5–40 min at 25, 30 or 37°C.
8. Repeat step 6.
9. Stimulation; re-suspend in 100 μl KG-buffer II or IV and incubate for 3–10 min at 25, 30 or 37°C. This step may be performed immediately after step 6, adding the substances under investigation directly to the stimulation buffer.
10. Remove supernatant and determine the amount of released catecholamines by liquid-scintillation counting.

11. Add 200 µl/well of 0.2% SDS to solubilize the cells and to determine the remaining amount of catecholamines.

2. Protocol 8: Measuring Exocytosis in Permeabilized Attached Cells

Solutions: see basic protocol 7

Steps (assay for exocytosis in chromaffin cells attached to culture plates)

1. Label cells (250 µl/well with a diameter of 20 mm) with radioactive noradrenaline for 2 h in the cell incubator.
2. Aspirate the labeling medium and chase cells for 1–2 h in DMEM.
3. Aspirate DMEM and wash cells with Krebs' buffer four times for 5 min.
4. Wash cells once with 200 µl KG buffer I or III.
5. Aspirate supernatant and add 200 µl of α-toxin or SLO dissolved in KG buffer I or III. Incubate with pore-forming toxins: α-toxin 20–30 min at 25, 30 or 37°C, SLO 5 min at 0°C or between 1 min and 2 min at 25, 30 or 37°C. About 30 HU/ml α-toxin or SLO, corresponding to 300 HU/10^7 cells is used.
6. Aspirate solution.
7. Incubate with substances to be tested dissolved in KG buffer I or III for 5–40 min at 25, 30 or 37°C.
8. Same procedure as in step 6.
9. Stimulation; add 200 µl KG buffer II and incubate for 3–10 min at 25, 30 or 37°C.
10. Remove supernatant and determine amount of released catecholamines by liquid-scintillation counting.
11. Add 200 µl/well of 0.2% SDS to solubilize the cells and to determine the amount of remaining catecholamines.

a) Commentary for Basic Protocols 7 and 8

Figure 4 gives representative experiments for Ca^{2+}-induced exocytosis from PC12 cells in suspension permeabilized either with various concentrations of α-toxin (*left*) or SLO (*right*). Steps 7 and 8 of basic protocol 5 were left out.

Depending on the types of experiments, ATP may be omitted during steps 5, 7 or 9. If the experiment does not work, check permeability with the trypan-blue test and Ca^{2+}-buffers with a Ca^{2+}-sensitive electrode (AHNERT-HILGER et al. 1989b; FÖHR et al. 1991).

IV. Regulation of Vesicular Transmitter Transporters in Permeabilized Cells

Transmitter uptake into secretory vesicles is an ATP-dependent process. So far, these studies are restricted to isolated secretory vesicles where intracellular substances necessary for regulation may be lost during purification. Neuroendocrine cells, such as PC12 cells, permeabilized with either pore-forming toxin can be used to study the regulation of transmitter storage without the

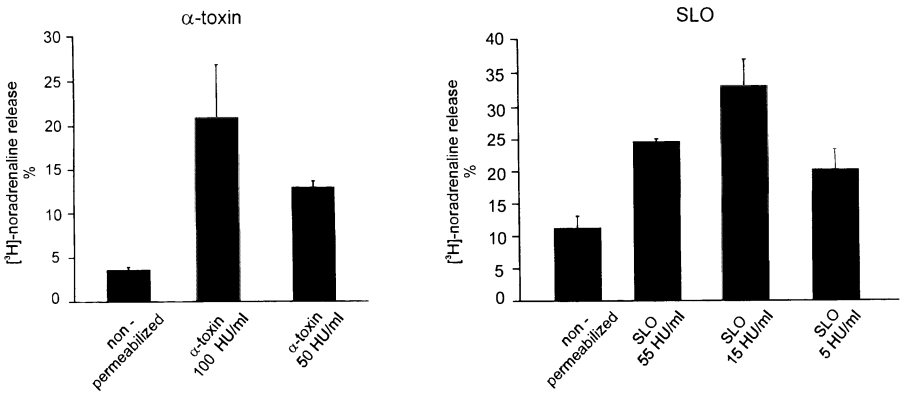

Fig. 4. Ca^{2+}-stimulated noradrenaline release from permeabilized PC12 cells. The experiment was performed as outlined in Sect. B. III. The free Ca^{2+}-concentration was 15 μM. Release in the absence of Ca^{2+} was subtracted. Values represent the means of three determinations ± the standard deviations.

need to purify secretory vesicles. Catecholamine uptake is driven by an ATP-dependent pH gradient across the vesicle membrane. The transporter is completely blocked by the addition of micromolar to nanomolar concentrations of reserpine. Two vesicular monoamine transporters have been cloned recently. In the rat, the vesicular monoamine transporter 1 (VMAT 1) is restricted to the adrenal medulla and, thus, is the transporter responsible for vesicular catecholamine storage in PC12 cells (Liu and Edwards 1997).

1. Protocol 9: Regulation of Vesicular Transmitter Transporters in Permeabilized Cells

Solutions

1. Buffer I. For 500 ml, dissolve 34.23 g sucrose (200 mM), 1.86 g KCl (50 mM), 3.024 g PIPES (20 mM), 0.7608 g EGTA free acid (4 mM) and 0.1017 g $MgCl_2$ (1 mM) and adjust to pH 7.0 using KOH
2. Buffer II. Use the same constituents as for buffer I. Add 2.87 g Mg^{2+}/ATP (2 mM) per 50 ml and adjust to pH 7.0 with KOH

Steps (assay of ATP-dependent uptake of catecholamines)

1. Grow cells on culture dishes. For PC12 cells, the best results were performed with one confluent 100-mm dish per 9–12 samples.
2. Remove medium from the cells. Re-suspend cells in Krebs'–4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (Hepes) and wash them twice with Krebs'-Hepes buffer by centrifugation (1 min at 2000 × *g* and 4°C).
3. Re-suspend pellet in ice-cold buffer I containing SLO or α-toxin.
4. Incubate samples with SLO for 5 min on ice, α-toxin samples for 10 min at 37°C (incubation time may be increased).

5. Centrifuge at 4°C and re-suspend in ice-cold buffer I.
6. Incubate for 20 min at 37°C to remove soluble cytosolic components.
7. Centrifuge 1 min at 2000 × *g* and 4°C and aspirate supernatant.
8. Re-suspend in buffer II containing ^{3}H-labeled transmitters, such as γ-aminobutyric acid or noradrenaline, and incubate for 20 min at 36°C.
9. Stop the reaction by diluting with 1 ml ice-cold buffer I followed by centrifugation for 1 min at 12000 × *g* at 4°C.
10. Remove supernatant and dissolve pellet in 0.2% SDS.
11. Use one aliquot to count radioactivity, the other for the determination of protein content.

a) Commentary for Basic Protocol 9

A representative experiment using varying α-toxin or SLO concentrations is given in Fig. 5. The assay is performed in suspension; however, as with exocytosis, an attached protocol may be also applied. In case the experiment does not work, check the permeability with the trypan-blue exclusion test, ATP-containing buffers or ^{3}H-labeled catecholamines, which may have been oxidized upon long storage. Using this approach, we recently showed that VMAT1 is regulated by the heterotrimeric G-protein subunit $G\alpha o_2$ (AHNERT-HILGER et al. 1998).

C. Chemicals Used in the Protocols

ATP (sodium salt), Sigma, A3377
BCA (disodium salt), Sigma, D-8284
BSA fraction V (pH 7.0), Serva

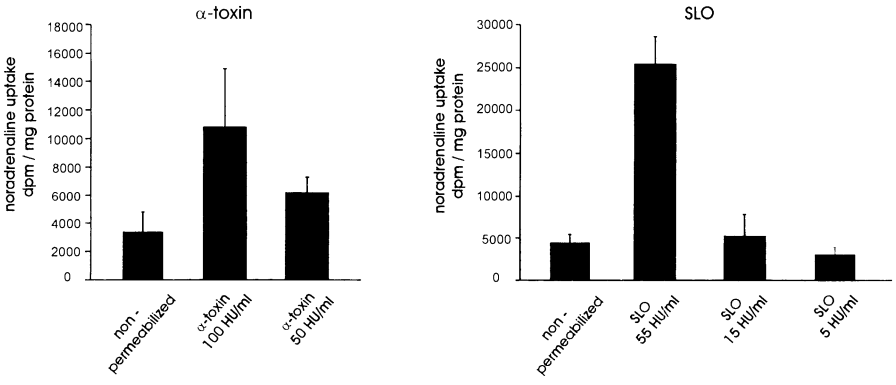

Fig. 5. Reserpine-dependent uptake of noradrenaline into permeabilized PC12 cells. The experiment was performed as given in Sect. B.IV. Uptake in the presence of 2 μM reserpine was regarded as unspecific and was subtracted. Values represent the means of three samples ± the standard deviations.

Digitonin, Sigma, D-1407
Goat-anti-antiserum Cy3-labeled, JACKSON DIANOVA
NGS, PAN Systems GmbH
^{3}H Noradrenaline, Amersham, TRA.584
Reserpine, Sigma, R-0875
Staphylococcus α-toxin (α-hemolysin) Sigma, H-9395 (not tested in the authors' lab)
SLO, Sigma, S-0149 (not tested in the authors' lab)
Trypan blue, Sigma, T-8154

D. Concluding Remarks

Pore-forming toxins like α-toxin and SLO are now established as cell-biological tools for the selective permeabilization of cells and cellular preparations. Using both α-toxin and SLO, it is possible to differentially permeabilize the plasma membrane by generating small or large holes, which allows study of intracellular processes either in the presence or absence of cytosolic proteins. In comparison with other permeabilization techniques, pore-forming toxins appear to be superior, at least for cells attached to culture dishes (including neurons in primary culture and cellular preparations like synaptosomes).

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CHAPTER 25

Heat-Stable Enterotoxin of *Escherichia Coli*

T. HIRAYAMA and A. WADA

A. Introduction

It is well known that certain strains of gram-negative bacteria *Escherichia coli* can cause intestinal and extraintestinal infections. Intestinal infection of *E. coli* leads to a variety of clinical syndromes, including watery and bloody diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome. LEVIN (1987) described several categories [enterotoxigenic *E. coli.* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli.* (EHEC), and enteroadherent *E. coli* (EAEC)] of diarrheagenic *E. coli*, and an additional category of enteroaggregative *E. coli.* (EAggEC) has been added. Among the most important diarrheagenic *E. coli* recognized is ETEC, a major cause of diarrhea in travelers, infant humans and animals in developed countries.

ETEC establishes its infection in the small intestine when the host ingests contaminated food or water. During its colonization, ETEC elaborates heat-labile enterotoxin (LT) or heat-stable-enterotoxin (ST). ETEC strains, when injected into ligated intestinal loops, behave like *Vibrio cholera*, causing fluid secretion and dehydrating diarrhea. Although the diarrhea caused by ETEC is typically milder than the diarrhea of cholera, the clinical features of ETEC infection are: watery diarrhea, nausea, abdominal cramps, and low-grade fever. LT and/or ST are known to be required for the virulence of ETEC in humans.

There are two serotypes of LT, LT-I and LT-II, on the basis of neutralization tests (HOLMES et al. 1986). Genes for LT-I and LT-II are located in plasmids (GLEYS et al. 1974) and in chromosomes (GREEN et al. 1983) of ETEC, respectively. LT-I shares a high amino acid sequence identity with cholera toxin (CT), and the three-dimensional structures of both toxins were shown to be very similar by X-ray crystallography (GIBBONS 1991; SIXMA et al. 1991). LT-I and CT bind strongly to ganglioside GM_1 and weakly to GD1b on the cell membranes of intestinal cells (FUKUTA et al. 1988). LT-I, but not CT, also binds to specific intestinal glycoproteins (HOLMGREN et al. 1982). In contrast, LT-II binds GD1a and GD1b (FUKUTA et al. 1988). Both LTs and CT exert their effects, at least in part, through the adenosine diphosphate ribosylation of a guanine-nucleotide-binding protein involved in the regulation of adenylyl cyclase. This covalent modification results in prolonged accumulation of intracellular cyclic adenosine monophosphate (cAMP) in intestinal tissue

(Moss and VAUGHAN 1989), leading to diarrhea through activation of various cAMP-dependent cellular functions. Of note, no definite role has been established for LT-II in diarrheal diseases.

ST is distinguished from LT because of its resistance to boiling at 100°C for 30 min. ST is excreted into the medium following a series of processing events that alter the length of the precursor; in contrast, LT remains in the periplasm. ST toxins have been classified into two structurally, functionally and immunologically unrelated types. One, STa or STI, is methanol soluble and active in suckling mice and piglets. The other, STb or STII, is methanol insoluble and was assumed, until recently, to be specifically active in weaned pigs. Recent work demonstrated STb enterotoxicity in mice, rats, rabbits and calves; STb uses a trypsin inhibitor to protect it from proteolysis in the intestine (WHIPP 1990). Both STa and STb are low-molecular-weight enterotoxins. Genes for STa and STb are carried on ETEC plasmids, having transposable elements Tn1681 (SO and MCCARTHY 1980) and Tn4521 (LEE et al. 1985), respectively. STa activates the membrane-bound form of intestinal guanylate cyclase and stimulates a secretory response by increasing the concentration of cyclic guanosine monophosphate (cGMP) within intestinal epithelial cells (FIELD et al. 1978; GUERRANT et al. 1980; RAO et al. 1980). STb has recently been purified, thus enabling researchers to define its structure and function (FUJII et al. 1991, 1994).

B. Heat-Stable Enterotoxin STa

I. Structure and Biological Properties of STa

STIa and STIb, which are genetically distinct, have also been named STp and STh, respectively, because they were originally found in *E. coli* isolated from pigs (and cows) and humans, respectively. STh and STp have been purified from the culture supernatants of human and porcine ETEC, respectively. Their primary sequences of 19 and 18 amino acids (Table 1: AIMOTO et al. 1982; TAKAO et al. 1983) have been determined with calculated molecular weights of 2048 Da and 1978 Da, respectively. Gene cloning also helped elucidate the molecular characteristics of STa (SO et al. 1980; MOSELEY et al. 1983). Production of STa is mediated by transmissible plasmids. Similar heat-stable enterotoxins are produced by other enteric pathogens, such as *Yersinia enterocolitica* (TAKAO et al. 1985b; YOSHINO et al. 1994), *V. cholera* non-O1 (TAKAO et al. 1985a), *V. cholerae* non-O1 Hakata (ARITA et al. 1991a), *V. cholerae* O1 (YOSHINO et al. 1993), *V. mimicus* (ARITA et al. 1991b) and *Citrobacter freundii* (GUARINO et al. 1989).

All heat-stable enterotoxins share the highly homologous sequence of 13 amino acid residues from Cys-6 to Cys-18 in the case of STh in Table 1. This conserved sequence of 13 amino acid residues in STa constitutes the active domain essential for full enterotoxicity (YOSHIMURA et al. 1985).

Table 1. Amino acid sequence of STa toxins from enteropathogens and disulfide-bond structures of STh. Note the conserved amino acids in all STa toxins

STa toxins	Amino acid sequence
E. coli	
STh (STIa)	NSSNY CC EL CC NPAC T GC Y
STp (STIb)	NTFY CC EL CCNPAC T GC Y
V. cholera	
O1 ST	FIKQVDENGNLID CC EI CC NPAC F GC LN
O1-Hakata ST	LID CC EI CC NPAC F GC LN
NAG-ST	ID CC EI CC NPAC F GC LN
V. mimicus ST	ID CC EI CC NPAC F GC LN
C. freundii ST	NTFY CC EL CCNPAC T GC Y
Y. enterocolitica ST	
a	QACDPPSPPAEVSSDWD CC DV CCNPAC A GC
b	KACDTQTPSPSEENDDW CC EV CCNPAC A GC

The crystallization of synthetic fully toxic analog of STp, Mpr^5-STp[5–17], where Mpr was β-mercaptopropionic acid and which consists of 13 amino acid residues from Cys-5 to Cys 17 in STp but is deaminated at its N-terminus (Kubota et al. 1989), was carried out (Ozaki et al. 1991). As revealed by X-ray crystallography, STa exhibits a right-hand-spiral structure with three β-turns fixed by three disulfide bonds. The second β-turn at residues Asn-11 to Cys-14 of STp or Asn-12 to Cys-15 of STh was amphiphilic and important for interaction with its receptor on intestinal epithelial cells. These four amino acids are conserved not only in all STs but also in guanylin (PNTCEICAYAACTGC; Currie et al. 1992), a mammalian analogue of STa. Disulfide bonds may play a critical role in determining the tertiary structure of these heat-stable enterotoxins. Analysis of nuclear magnetic resonance spectra of synthetic STa and native STa showed that synthetic STa had the same three dimensional structure as native toxin, including the disulfide-bond positions.

The biological activities of synthesized STa and its analogs were examined by the fluid-accumulation test in suckling mice. The core sequences (STh[6–18] and STp[5–17]) have the same toxic activities as the entire sequences of STh and STp, respectively, and are more stable to heat treatment than native toxins. The minimum effective doses of the synthetic peptides were 0.4 pmol for STh[6–18] and 0.5 pmol for STp[5–17], which corresponded to those of native

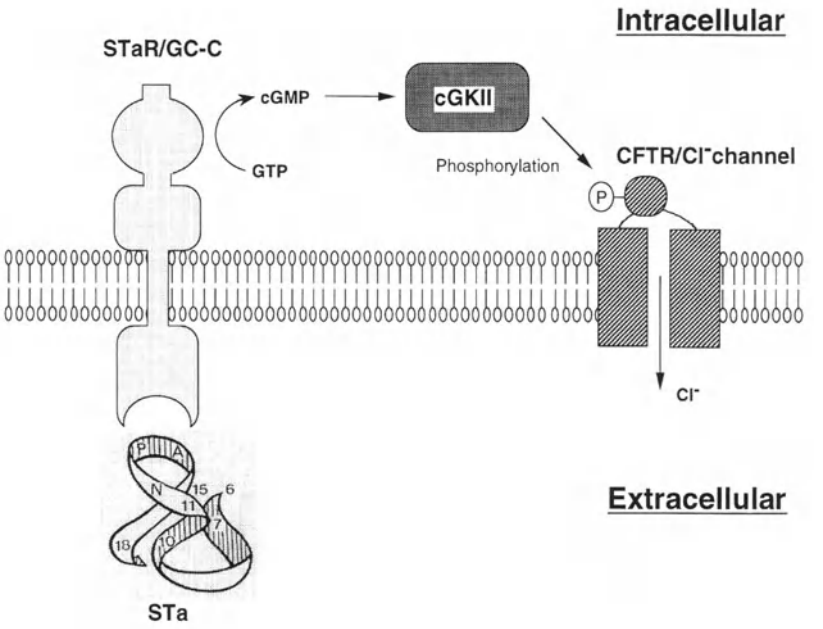

Fig. 1. Binding and cell signaling of STa on its receptor, STaR

STh and STp, respectively. These results imply that the core sequences with 13 amino acid residues, linked intramolecularly by three disulfide bonds from the cysteine residue near the N-terminus to the cysteine residue near the C-terminus, are responsible for the enterotoxigenic activity of STa and form spatially compact and very stable structures. Moreover, when these synthetic peptides were treated with antiserum raised against purified native STh, their biological activities were neutralized in a manner similar to that for native STh and STp.

A Corey-Pauling-Koltun model and schematic ribbon drawing (Fig. 1) of a short analog of STh with 13 amino acid residues (YAMASAKI et al. 1988) exhibited a twisted "8" structure of the main chain, which seems to be important for expression of the enterotoxigenic activity of STh and for stabilization of spatial structure of the peptide chain from Cys-7 to Cys-15.

II. Receptor for STa

Analysis of the receptor for STa will help determine the early events involved in intoxication of target cells and lead to better understanding of the mechanism of cellular intoxication by STa. Immunohistochemical study has shown that specific high-affinity STa receptors (STaR/GC-C) are concentrated in villus enterocytes but not in crypts (ALMENOFF et al. 1993). As shown in Fig. 1, the binding of STa to STaR activates the intracellular guanylyl-cyclase (GC)

domain of the receptor, causing substantial increases in the intracellular cGMP content (FIELD et al. 1978; HUGHES et al. 1978; NEWSOME et al. 1978). The resulting increase in cGMP stimulates net fluid secretion through activation of apical Cl^- channels in parallel with inhibition of coupled NaCl transporters (FIELD et al. 1989). The cystic fibrosis (CF) transmembrane-conductance regulator, an epithelial Cl^- channel mutated in CF patients (COLLINS 1992; WELSH and SMITH 1993), appears to be involved in the Cl^- secretory response to STa and cGMP analogues acting through the cGMP-dependent protein kinase II (cGKII; PFEIFER et al. 1996; VAANDRAGER et al. 1997), as evidenced by the absence of this response in CF intestine (BAXTER et al. 1988).

STaR has been reported to be a distinctly different protein from GC. STa-binding activity of STaR was separated chromatographically from GC activity (KUNO et al. 1986). Using low-stringency polymerase chain reaction (PCR) with degenerate oligonucleotides based on a highly conserved region within the GC domains, however, SCHULZ et al. cloned a novel GC (STaR/GC-C) in rat intestine, which possessed STa-binding activity (SCHULZ et al. 1990). To date, rat, pig and human STaRs have been reported (DE SAUVAGE et al. 1991; SINGH et al. 1991; WADA et al. 1994). The deduced sequence of the pig STaR has 1073 amino acids. Based on hydrophobicity analysis, the N-terminal 23 amino acid residues represent a signal peptide, with the mature protein containing 1050 amino acid residues (approximately 121 kDa). The deduced amino acid sequence of the pig STaR shows overall sequence similarities of 87% and 82% with those of the human and rat receptors, respectively. Its sequence has 76% and 71% identity in the extracellular region (amino acids 1–410) and 95% and 90% identity in the intracellular region (amino acids 432–1050) with the human and rat receptors, respectively. These results imply that intracellular amino acids are conserved to a greater extent than extracellular amino acids; further, pig STaR is more closely related to the human receptor than to the rat receptor.

Expression of the STaR gene in COS-7 cells resulted in elevated GC activity. Moreover, following transfection of cells with the STaR gene, STa, but not other GC-activating peptides (natriuretic peptides), caused marked elevations of cGMP levels through its specific binding to the transfected cells. STa failed to elevate cGMP in non-transfected cells or in cells transfected with natriuretic peptide receptor (NPR)-A (GC-A), another membrane-associated GC (SCHULZ et al. 1990). These results show that STaR is an STa receptor.

To examine the role of STaR in acute diarrhea, several synthetic STa analogs from human and porcine strains of *E. coli*, *V. cholerae* non-O1, and *Y. enterocolitica* were examined for both elevation in GC activity in CHO cells transfected with STaR gene and effects on fluid secretion in a suckling-mouse model. The effects on cGMP in STa-exposed recombinant cells correlated well with increase in fluid secretion (WADA et al. 1994). Accordingly, STaR was a functional receptor for STa-mediated diarrhea.

Northern-blot analysis demonstrated that the small and large intestine are the primary sites of transcription of the 4-kb mRNA encoding STaR. In the

small intestine, binding of radiolabeled STa was maximal in the villus preparations and gradually decreased along the villus-to-crypt axis. In situ hybridization also demonstrated a clear signal in the villus cells with no apparent signal in the crypt cells, lamina propria or muscularis. Expression of STaR was greatest after enterocytes leave the proliferative cycle and enter villi (COHEN et al. 1992; ALMENOFF et al. 1993). This topological arrangement suggests that there are autocrine or paracrine pathways by which STaR interacts with endogenous ligands. Expression of STaR mRNA in other than intestinal cells, was detected in mouse liver (SWENSON et al. 1996), rat adrenal gland, and bovine trancheal mucosa (SCHULZ et al. 1992). PCR amplified STaR gene from rat olfactory mucosa and human airway epithelial cells. Although STaR was not detected in rat brain by PCR methods, in the process of cloning membrane-associated GC, STaR complementary DNA (cDNA) clones were obtained from a brain cDNA library (SCHULZ et al. 1992). Thus, very small amounts of STaR mRNA are expressed in several mammalian cell types. LANEY et al. found that, in the regenerating liver of the hepatectomized rat, STaR mRNA at 24 h after partial hepatectomy increased 30–40-fold, attaining intestinal levels. and radiolabeled STa binding to cells membranes also increased over normal levels at 24 h but fell to near normal levels by 31 h (LANEY et al. 1994). Similarly, high levels of STaR expression were detected in the rapidly growing liver of the newborn rat (LANEY et al. 1992). Cell-separation experiments revealed that the nonparenchymal cell fractions of regenerating liver contained four times as much STaR as purified hepatocytes. Immunohistochemistry confirmed these findings (SCHEVING and RUSSELL 1996). Thus, STaR may have an important role during liver generation; at the molecular level, the functional role in the liver is still not clear.

Recently, MANN et al. and SCHULZ et al. generated STaR-knockout mice (MANN et al. 1997; SCHULZ et al. 1997). Intestinal mucosa GC activity was about 16-fold higher in wild-type than in STaR-null mice, suggesting that STaR is the major cause of GC activity in intestine. In agreement with this hypothesis, radiolabeled STa did not bind to intestinal membranes from null mice. In heterozygous mice, radiolabeled STa binding and STa-mediated GC activity were about one-half those found in wild-type mice; they were absent in STaR-null mice. These results suggest that STaR completely accounts for the STa-induced elevations of cGMP, although there is evidence (including kinetic analysis of STa-binding and receptor cross-linking studies) for the existence of other STa receptors (HIRAYAMA et al. 1992).

The response of the STaR-null mice was assessed in the suckling-mouse assay. While exposure to STa caused significant intestinal secretion in wild-type and heterozygous mice, no secretory response was seen in STaR-null mice. Challenge with permeable analogs of either cGMP (the second messenger for STa) or cAMP, another potent secretagogue, resulted in secretion in all mice, indicating that the downstream secretory pathway was intact. Accordingly, these results showed that STaR is necessary for the diarrheal response induced by STa.

STaR has structural features in common with other members of mammalian membrane-associated GCs, including an extracellular peptide-hormone- or toxin-binding domain, a single transmembrane domain and an intracellular signaling domain comprised of a kinase-homology domain and a GC-catalytic domain. As for the role of the kinase-homology domain, RUNDER et al. demonstrated that its deletion produces a constitutively active mutant, suggesting that this domain serves an autoinhibitory function (RUNDER et al. 1995). They proposed that binding STa to STaR promotes a conformational change across the cell membrane, which removes the inhibitory effects of the kinase-homology domain and promotes an interaction between cyclase domains, leading to receptor activation. In agreement with this hypothesis, the kinase-homology domain of another membrane-bound GC, NPR-A also acts as a negative regulator of signaling (CHINKERS and GARBERS 1989). Thus, kinase-homology domains of membrane-associated GCs might function in regulation of the GC domain. However, the kinase-homology domain of STaR cannot effectively regulate ligand activation of NPR-A (KOLLER et al. 1992). Therefore, based on this finding and on the limited homology of the kinase-homology domains (~35%) of STaR and NPR-A, it was proposed that there may be differences in the signaling mechanisms between these two receptors.

The extracellular domain of STaR has multiple N-linked carbohydrates. Antibodies raised against the C-terminal sequence of STaR identified two major proteins (140 kDa and 160 kDa) in 293 cells expressing STaR; these proteins bound to wheat-germ lectin–Sepharose. *N*-glycosidase-F treatment converted both forms to a single 120-kDa protein, the same size predicted from the deduced amino acid sequence (HIRAYAMA et al. 1993; VAANDRAGER et al. 1993). These N-linked carbohydrates in the extracellular domain of STaR may serve to enhance protease resistance in intestine and/or ensure the proper conformation for STa binding.

DESHMANE et al. examined equilibrium- and kinetic-binding characteristics of STaR expressed in COS-7 cells to determine if this receptor exhibited multiple affinities for STa. Scatchard analysis of equilibrium binding yielded curvilinear isotherms consistent with the presence of high (RH, pM)- and low (RL, nM)-affinity sites, and a ligand-induced conversion from a higher (RL1)- to a lower (RL2)-affinity state. Occupancy of the low-affinity sites was coupled to ligand-induced catalytic activation (DESHMANE et al. 1995). The ligand-binding and catalytic-activation properties of a series of intracellular deletion mutants of STaR were examined to identify the structural domains responsible for high- and low-affinity sites and the mechanism of the ligand-induced shift to low-affinity sites. These studies demonstrated that the extracellular and transmembrane domains are sufficient for expression of high-affinity binding sites, and cytoplasmic domains of STaR were not required. In addition, the cytoplasmic juxtamembrane and kinase-homology domains were required for the ligand-induced shift to low-affinity sites (DESHMANE et al. 1997).

To define the domain of STaR responsible for binding to STa, the extracellular region of STaR was examined by site-directed mutational analysis

(WADA et al. 1996a). A series of mutations was generated at two regions in the extracellular domain of STaR: (1) the HC region, which is highly conserved among rats, humans and pigs, and (2) the ENTM region, which corresponds to a region in the extracellular domain near the transmembrane region. The first region, encompassing residues from Arg-91 to Asn-155, has 92% and 91% identity in the human and rat STaRs, respectively, whereas the predicted amino acid sequence of the complete extracellular domain of pig STaR shows 76% and 71% identity, respectively. The second region, from Leu-274 to Leu-409 of the extracellular domain, is near the transmembrane domain and has 136 amino acid residues. In most STaR mutants, charged amino acids (especially Asp and Glu) were replaced by neutral residues (Ala or Gly), since charged residues are generally located on the molecular surfaces of proteins and, therefore, may interact with ligands. Eighteen STaR mutant proteins were examined for binding affinity to STa, GC activity and expression on 293 T cell surfaces. The results show that mutation of Arg-136–Leu137–Met-138 to Gly-Leu-Val in the HC region and of Asp-347–Asn348 to Ala-Ser in the ENTM region had significant effects on both binding to STa and GC activity (WADA et al. 1996b).

In the membrane-associated GC family, adenine nucleotides appear to be important in coupling ligand-receptor interactions to enzyme activation. Using magnesium as the cation cofactor in the presence of 1 μM STa adenine nucleotides that were not substituted at the 2 position activated GC with the following rank order of potency: adenosine triphosphate (ATP)γS > ATP > adenosine monophosphorothioate. STaR was maximally activated two- to threefold by ATPγS in the presence of STa in a concentration-dependent fashion (GAZZANO et al. 1991). In contrast, under identical conditions, the 2-position-substituted adenine nucleotide analogues 2MeSATP and 2ClATP inhibited GC activity. However, when manganese was used as the substrate cofactor, adenine-nucleotide-dependent inhibition of STaR was preferentially observed with all ATP analogues inhibiting GC activity of STaR. Considering that the GC activity of STaR was inhibited in a non-competitive manner, these activating and inhibitory effects of adenine nucleotides on STaR may be mediated by separate pathways (PARKINSON et al. 1994).

In the T84 colonic carcinoma intestinal cell line, phorbol esters have an additive effect on STa-mediated GC activity, consistent with a possible role for phosphorylation by protein kinase C (PKC; WEIKEL et al. 1990). It is notable that the activation effect of phorbol myristate acetate (PMA) on STa-mediated GC activity of STaR is the opposite of the inhibitory effects of PMA on NPR-A-stimulated GC activity. CRANE et al. demonstrated that phosphorylation of STaR was blocked by a synthetic peptide, KPRRVASYKKG, corresponding to the sequence around Ser-1029 in the C-terminal tail of STaR, suggesting that the putative phosphorylation site is Ser-1029 (CRANE and SHANKS 1996). To characterize Ser-1029 in STaR as a consensus sequence of phosphorylation site by PKC, two mutants, mS1029A (with replacement of Ser-1029 with Ala-1029) and CΔ1029 (which lacks 22 amino acids, including Ser-1029) were generated. Preincubation of the wild-type STaR transfectant

with 1 mM PMA caused additional STa-mediated GC activation compared with control, whereas the mS1029A- and CΔ1029-transfected cells did not show a similarly enhanced GC activation by PMA. Using ^{32}P-orthophosphate metabolic labeling to look at phosphorylation, the immunoprecipitated wild-type STaR (but not mS1029A and CΔ1029) incorporated significant radioactivity. These results suggest that the effect of PMA on wild-type STaR transfectants might be caused by phosphorylation of Ser-1029 (Wada et al. 1996a).

C. STa-like Heat-Stable Enterotoxin

Another ST, EAST1, which is genetically and immunologically distinct from STa and STb, was elaborated by an EAggEC strain (Savarino et al. 1991, 1993). EAST1, encoded by the astA plasmid-associated gene, consists of 38 amino acids (molecular weight 4100 Da), including four Cys residues (Fig. 2). EAST1 shows significant homology (TACASC in the EAST1 sequence) with the enterotoxic domain of STa and with guanylin; it exhibited enterotoxigenic activity and increased ileal-tissue cGMP levels. A synthetic peptide composed of residues 8–29 of EAST1 also showed enterotoxic activity. Based on the cGMP data and the sequence homology to STa and guanylin, it is predicted that EAST1 stimulates the particulate form of GC through the same receptor-binding region as STa and guanylin. Little is known about either the contribution of EAST1 to diarrheal disease caused by EAggEC infection or the structure–function relationship. The distribution and prevalence of the EAST1 gene in selected groups of bacterial enteropathogens were determined by colony hybridization; 100% of 75 O157:H7 EHEC, 41% of 227 EAggEC, 41% of 149 ETEC, 22% of 65 EPEC and 38% of 47 *E. coli* stool isolates from asymptomatic children hybridized with an EAST1 DNA probe. None of 55 EIEC, 12 of *Y. enterocolitica* and 20 of *V. cholerae* non-O1 strains were EAST1-probe-positive. The gene encoding EAST1 is broadly distributed among diarrheogenic *E. coli*, suggesting that EAST1 is not restricted to EAggEC (Savarino et al. 1996).

D. Heat-Stable Enterotoxin STb

I. Structure of STb

The genes encoding STb were cloned, and the nucleotide sequences were determined (Lee et al. 1983; Picken et al. 1983). In 1991, Fujii et al. (1991)

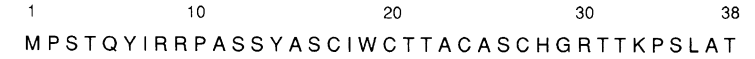

Fig. 2. Predicted amino acid sequence of EAST1 expressed by *ast*A gene in enteroaggregative *Escherichia coli.* (*EAggEC*)

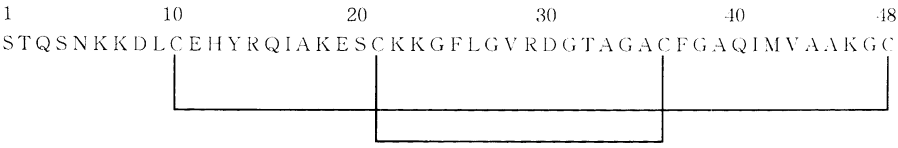

Fig. 3. Amino acid sequence and disulfide-bond structures of STb of enterotoxigenic *Escherichia coli* (*ETEC*)

established the purification procedure for STb and examined its chemical and biological properties. There is no amino acid sequence homology between STa and STb. It was found that STb consists of 48 amino acid residues with an isoelectric point of 9.7 and a molecular weight of 5104 Da (Fig. 3). The amino acid sequence of STb corresponds to the inferred C-terminal amino acid sequence of STb predicted from the DNA sequence, which encodes a 71-amino acid precursor protein (Lee et al. 1983). The precursor protein is cleaved by a signal peptidase to remove a 23-amino-acid signal peptide and is then translocated to the periplasm where, prior to secretion, two intramolecular disulfide bonds between Cys-10 and Cys-48 and between Cys-21 and Cys-36 are formed by an action of DsbA, the *dsbA* (*ppfA*) gene product (a periplasmic protein responsible for disulfide-bond formation in some periplasmic and outer membrane proteins of *E. coli*; Bardwell et al. 1991; Akiyama et al. 1992), resulting in resistance to proteolysis (Okamoto et al. 1995). Treatment of STb with reductant abolishes the enterotoxicity of STb, suggesting that disulfide bonds play a critical role in its tertiary structure and are essential for signal transduction. Substitution of nine basic amino acid residues (including one histidine, two arginine, and six lysines) by site-directed mutagenesis caused a marked reduction in enterotoxicity, especially when Lys-22, Lys-23, Arg-29 and Asp-30, which are in a loop defined by Cys-21 and Cys-36, are replaced (Drefus et al. 1992; Fujii et al. 1994).

II. Biological Function of STb

Crude toxin preparations of STb induced fluid secretion within 30 min, with a maximal response between 3 h and 6 h (Kenedy et al. 1984). The only animal responsive to STb is the pig. However, in the presence of protease inhibitors, STb was found to induce intestinal fluid secretion in rats and infant-mouse intestinal loops (Whipp 1987, 1990), indicating that the host-response specificity of STb is not dependent on special properties of pig intestinal cells.

To achieve its enterotoxigenic effect on intestinal cells, STb binds to intestinal epithelial cells through its receptor(s). Using cross-linking studies with ^{125}I-labeled STb, Hitotsubashi et al. reported that STb specifically binds to a 25-kDa protein of mouse intestinal cell membranes (Hitotsubashi et al. 1994). However, ^{125}I-STb-binding and immunofluorescence studies show that

STb binds with relatively low affinity (10^{-5} M) to membrane lipids of cultured intestinal epithelial cells and becomes stably associated with the lipid bilayer, enhancing its ability to traverse the membrane bilayer and enter into the cytoplasm (Chao and Dreyfus 1997). Binding and subsequent internalization of ^{125}I-STb were not affected by treatment of the cells with trypsin, endoglycosidase F/peptide *N*-glycosidase F, *V. cholerae* neuraminidase, tunicamycin, or 5 mM sodium chlorate, which blocks sulfation of surface proteoglycans, indicating that cell-surface proteins or carbohydrates did not function as STb receptors. Using biotinylated STb, Rousset et al. demonstrated its binding to jejunum, duodenum, ileum, cecum, colon, liver, lung, spleen and kidney of piglets (Rousset et al. 1998). Metaperiodate treatment of jejunal sections diminished STb binding; protease treatment did not affect the toxin binding. Reduced STb binding was observed following treatments with ceramide glycanase, α-glucosidase and neuraminidase, but not with *N*- and *O*-glycosidases and several exoglycosidases. STb binding to pig intestinal cells appears to involve a molecule consisting of a ceramide moiety, a terminal neuraminic acid and/or α-linked terminal glucose residues(s). The structure and function of receptor molecule(s) for STb are still unknown.

Weikel and Guerrant (1985) indicated that STb induced no change in Cl^- or Na^+ fluxes, as judged by use of labeled ^{22}Na and ^{36}Cl. The removal of Ca^{2+} and the addition of lanthanum did not inhibit fluid secretion due to STb, suggesting that this secretion is calcium independent. However, STb increased in the concentration of free Ca^{2+} found in the cytoplasm in renal and intestinal epithelial cultured cells. It was caused by influx of extracellular Ca^{2+}, not by Ca^{2+} release from intracellular Ca^{2+} stores. Dreyfus et al. showed that STb-induced intracellular Ca^{2+} concentration was not inhibited by drugs that blocked voltage-gated Ca^{2+} channels, including nitrendipine, verapamil (L-type), ω-conotoxin (N-type) and Ni^{2+} (T-type) or by prior treatment of the cells with thapsigargin or cyclopiazonic acid, which block internal Ca^{2+} stores (Dreyfus et al. 1993). Somatostatin and pertussis toxin inhibited the STb-induced increase in intracellular Ca^{2+} concentration. STb-induced increases in Ca^{2+} levels in the cytoplasm appear to be due to opening of a G-protein-linked receptor-operated Ca^{2+} channel in the plasma membrane. STb induced the contraction of the mouse ileum and increased the amount of prostaglandin E2 (PGE2) in the ileum without affecting the intracellular concentration of cyclic nucleotides (Hitotsubashi et al. 1992). Prostaglandin-synthesis inhibitors, such as aspirin and indomethacin, significantly reduced the enterotoxicity of STb. STb stimulated the release of both PGE2 and serotonin (5-hydroxytryptamine, 5-HT) from the pig intestinal mucosa, suggesting an effect on the enteric nervous system (Peterson and Whipp 1995). The release of serotonin from rat basophilic leukemic cells, induced by STb, is independent of extracellular Ca^{2+} and is completely blocked by pretreatment of the cells with pertussis toxin, indicating a role for a pertussis-toxin-sensitive G protein in serotonin release by STb (Harville and Dreyfus 1996). However, it is not clear whether serotonin and PGE2 are formed by direct or indirect effects of

STb. The role of arachidonic-acid metabolites and neurohormones in intestinal secretion induced by STb remains unclear. Further studies on the mechanism by which STb causes intestinal secretion are needed.

E. Concluding Remarks

Even though the structures of heat-stable enterotoxins produced by ETEC strains are already determined, many other questions about signal-transduction pathways of these toxins remain. For example, it would be important to get more information about the conformational and spatial structural changes of STaR/GC-C occurring upon activation by STa. One important topic is the elucidation of the precise role of the kinase homology domain of GC and its regulation by cytosolic substance(s) to maintain the cGMP concentration. Those studies will not only give insights into the actions of the heat-stable enterotoxins but will provide important additional information about "physiological" signaling (via GC receptors) by hormonal factors. Much less is known about STb. More work is needed to understand the mechanisms by which STb acts on intestinal epithelial cells. The receptor for STb is still unknown, and the precise roles of PGE2 and serotonin in the action of STb have to be determined. It is suggested that further clarification of the structure–function relationship of the STs and their receptors will have a major impact on the development of more effective prevention of ST-mediated diarrhea.

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CHAPTER 26

Superantigenic Toxins

B. FLEISCHER

A. Summary

"Superantigens" is the designation for a heterogeneous group of proteins that use a common, extremely efficient mechanism of T-lymphocyte stimulation. They bind to major histocompatibility complex (MHC) class-II molecules on antigen-presenting cells (APCs) and to various parts of the T cell receptor (TCR) on $CD4^+$ and $CD8^+$ T cells, thus mimicking the recognition of specific antigens. The prototype superantigen is staphylococcal enterotoxin (SE) B, member of a family of genetically related pyrogenic exotoxins (PET) produced by *Staphylococcus aureus* and *Streptococcus pyogenes*. This principle of T-lymphocyte stimulation has evolved independently because of infectious pathogens.

Investigations by a number of laboratories have elucidated the unusual mechanism of T-lymphocyte stimulation and have shown that the pathogenic effects of these molecules are due to their ability to stimulate a large fraction of T cells. Consequences of confronting the body with superantigenic toxins are shock (mediated by a massive liberation of cytokines from cells of the immune system) and immunosuppression, probably caused by an uncoordinated activation of the immune system and a massive deletion of T cells.

B. Introduction

Antigen recognition by T lymphocytes is one of the most sensitive assays in biology. With its antigen receptor, the T cell can respond to a single antigenic peptide presented by an APC within 1 min. This unique sensitivity is provided by a set of molecular mechanisms that intracellularly amplify the initial signal given by one or only a few TCRs. Superantigens have been adapted via evolution to use the T cell machinery to become effective T cell stimulators. Although there are different types of superantigens, which have been independently generated several times via evolution and which are completely unrelated to each other, they all use the same molecular mechanism: they bind to MHC class-II molecules on APC and to the TCR. Thus, by closely mimicking antigen recognition, they use most of the amplification mechanisms the T cell uses normally. Thus, a hallmark of all superantigens is their enormous

potency: they are active in concentrations of 10^{-9} M. This means that a few picograms per milliliter can still effectively stimulate T cells in culture. Another characteristic feature of superantigens is their use of variable determinants on molecules of the immune system; all known superantigens bind to the variable part of the TCR β-chain (Vβ). Thus, each superantigen stimulates a particular fraction (up to 30%) of the T cell repertoire. On the MHC-II molecule, there are different binding sites used by different superantigens; some superantigens even involve the antigenic peptide in their binding.

This principle of T-lymphocyte stimulation has independently evolved several times. Infectious pathogens as different as *S. aureus*, *Yersinia pseudotuberculosis*, *Mycoplasma arthritidis* and mouse mammary-tumor viruses produce superantigens that are apparently completely unrelated to each other. Obviously, this very special mechanism of T cell stimulation has been developed independently by such different pathogens via evolution. Thus, it is likely that additional pathogens will also use it. Therefore, in recent years, there have been surprisingly many reports describing novel superantigens in various viruses, bacteria and protozoa. With one exception, the lectin *Urtica dioica* agglutinin of the stinging nettle (Galelli et al. 1993), all molecules that have been proposed to be superantigens are produced by infectious pathogens. The long list of candidate superantigens includes bacteria, protozoa and viruses, some of which are listed in Table 1. In most cases, the evidence is still indirect or the initial report has not been confirmed by an independent study. In this review, I will limit the discussion to superantigens of bacterial origin.

C. PETs of *S. Aureus* and *S. Pyogenes*

I. Genes and Molecules

The PETs produced by these two genera of gram-positive cocci comprise a polymorphic family of genetically related toxins consisting of the SEs, the toxic-shock-syndrome-toxin-1 (TSST-1) produced by *S. aureus* and the streptococcal PET (SPE) A produced by *S. pyogenes*. *S. aureus* is widespread among man and animals, and many of these toxins are also present in *S. aureus* strains from other species. They are heat-stable and protease-resistant molecules of 22-kDa to 28-kDa molecular mass.

Historically, the SEs were defined by their ability to cause emesis and diarrhea in humans and monkeys. They are a major cause of food poisoning worldwide. Currently, eight major types (SE A, B, C, D, E, G, H and I) are recognized, of which SEs A–E were originally serologically defined; SEs G–I were named after cloning and purification from individual strains. TSST-1 was initially designated SEF but was later renamed TSST-1 due to its association with toxic-shock syndrome and because the initial finding of emetic activity could not be confirmed (Reiser et al. 1983).

The SPEs are usually discussed separately from the SEs, because they are derived from another genus and because an enterotoxic activity has not been

Table 1. Some candidate superantigens produced by microorganisms

Producing pathogen	Protein	Other activities	Reference
Staphylococcus aureus	Enterotoxins A–I		See text
	Toxic-shock-syndrome toxin 1		See text
	Epidermolytic toxins A, B	Proteases	See text
Streptococcus pyogenes	Erythrogenic toxins A, C SSA		See text See text
	SMEZ		Kamezawa et al. 1997
	Erythrogenic toxin B	Protease	See text
	M protein	Receptor	See text
	MF	Nuclease	See text
Clostridium perfringens	Enterotoxin	Pore former	Bowness et al. 1992; McClane 1994; Krakauer et al. 1997
Pseudomonas aeruginosa	Exotoxin A	ADP–ribosyltransferases	Legaard et al. 1991
Yersinia pseudotuberculosis	YPM		See text
Y. enterocolitica	Undefined		Stuart and Woodl 1992
Mycobacterium tuberculosis	Undefined		Ohmen et al. 1994
Mycoplasma arthritidis	Soluble MAS		See text
Toxoplasma gondii	Undefined		Denkers et al. 1994

ADP, adenosine diphosphate; *MAS*, *M. arthritidis* superantigen; *MF*, mitogenic factor; *SMEZ*, streptococcal mitogenic exotoxin Z; *SSA*, streptococcal superantigen; *YPM*, *Y. pseudotuberculosis* mitogen.

seen in them. However, since such activity can be detected only in man or monkeys, it is not clear if this putative difference has been sufficiently investigated. From a genetic point of view, the separation of the SPEs from the SEs is not appropriate, since some SPEs are more related to SEs than to other SPEs. The members of the SE family can be divided into subgroups according to their sequence relatedness (Betley et al. 1992; Munson et al. 1998). They are listed in Table 2. Homologies vary considerably within the groups. A common feature of the PETs (except for TSST-1 and SE I) is a central disulfide loop. Although some PETs, such as TSST-1 and SPE C, share little

Table 2. Members of the enterotoxin family of pyrogenic exotoxins of *Staphylococcus aureus* and *Streptococcus pyogenes* (Betley et al. 1992; Munson et al. 1998)

Toxin	Molecular weight (Da)	Amino acids	Subgroup	% Sequence identity to	
				SE A	SE B
SE A	27100	233	SE A	100	28
SE B	28336	239	SE B	28	100
SE C1	27531	239	SE B	23	66
SE C2	27589	239	SE B	24	66
SE C3	27563	239	SE B	25	66
SE D	26360	228	SE A	52	36
SE E	26425	230	SE A	82	32
SE G	27107	233	SE B	20	39
SE H	25120	218	SE A	31	25
SE I	27043	218	SE A	28	19
TSST-1	22049	194	TSST	n.s.	n.s.
SPE A	25787	251	SE B	25	50
SPE C	24354	235	SPE C	n.s.	n.s.
SSA	26892	234	SE B	27	60
SMEZ[a]	25254	209	n.a.	n.a.	n.a.

n.a., not available; *n.s.*, not significant; *SE*, staphylococcal enterotoxin; *SMEZ*, streptococcal mitogenic exotoxin Z; *SPE*, streptococcal pyrogenic exotoxin; *SSA*, streptococcal superantigen; *TSST*, toxic-shock-syndrome toxin.
[a] Genetic relationship to SE and SPE not clear.

similarity with any other PET, there is sufficient sequence homology among all PETs to suggest that streptococcal and staphylococcal PETs have evolved from a single ancestral gene. This agrees with the finding that the genes for many of the PETs are encoded on mobile genetic elements; those for SE A, SE E, SPE A and SPE C are carried within bacteriophage DNA and those for SE D are on plasmid DNA (Weeks and Ferretti 1984; Goshorn and Schlievert 1989; Betley et al. 1992). TSST-1 and SSA are encoded on the chromosome, within heterologous DNA insertions. The streptococcal superantigen (SSA) is more related to SE B and SE C than to other streptococcal PETs, suggesting an intergeneric transfer; it appears to have been acquired by several different clonal lineages of *S. pyogenes* during this century (Reda et al. 1994). In *S. aureus*, the production of the SE is regulated by different loci: the accessory gene regulator (*agr*) the staphylococcal accessory regulator (*sar*) and the extracellular protein regulator (*xpr*), which regulate the production of a number of different exoproteins. The regulation of SPE production in *S. pyogenes* is less well understood.

A characteristic feature of SE and SPE is their extensive polymorphism; for some toxins, there are many alleles known that differ in only a few amino acids. For example, three subtypes of SE C have been numbered (SE Cs 1–3), and several variants of SE C from *S. aureus* strains derived from humans, dogs,

cattle and food have been cloned (MARR et al. 1993). The variants differ only in a few amino acids but may differ in their biological activities (BOHACH 1997). Several isolates of SE C3 that are serologically indistinguishable differ in several mutations. SE A and SE E have 82% homology and show strong serological cross-reaction; thus, SE E could be classified as a variant of SE A. TSST-1 produced by *S. aureus* strains associated with humans has a pI of 7.0, whereas *S. aureus* isolates from sheep produce the variant TSST-0, with a pI of 8.6 (Ho et al. 1989). TSST-0 differs in only seven amino acids from TSST-1 but differs dramatically in its biological activity (MURRAY et al. 1994). Four different variants of SPE A have been described (NELSON et al. 1991), as have three variants of SSA (REDA et al. 1994).

The frequency of enterotoxin producers among clinical *S. aureus* isolates has been determined in several studies (LEHN et al. 1995). Depending on the origin of the isolates, approximately 40–65% of *S. aureus* strains produce a PET. Most commonly, SE A is produced (20–25% of strains), followed by TSST-1 (13–23%), SE B (7–13%), SE C (5–9%), SE D (3–6%) and SE E (>1%). Two enterotoxins, usually SE A + TSST-1 or SE A + SE D, are produced by 5–8% of strains, which is a much higher frequency than would have been expected from respective individual frequencies. Isolates producing toxins, such as SEs G–I, are extremely rare. Data about the production of SPE A and SPE C by *S. pyogenes* strains isolated from various sources vary considerably, reflecting the variability of clinical sources and the sensitivity of detection, since *S. pyogenes* produces lower amounts of SPE than *S. aureus* does of SE. Information about the purification of the natural proteins has been reviewed (ALOUF et al. 1991).

For several members of the SE family (SE A, SE B, SE C2 and TSST-1), crystal structures are now available (SWAMINATHAN et al. 1992; PRASAD et al. 1993; ACHARYA 1994; PAPAGEORGIOU et al 1995; SCHAD et al. 1995). More recently, the crystal structures of SEB and TSST-1 complexed with the human MHC-II molecule HLA-DR1 have been determined and have – together with a large number of studies using mutated toxins or class-II molecules – illustrated the molecular mode of binding of the toxins to the class-II molecule (JARDETZKY et al. 1994; KIM et al. 1994).

In spite of their low level of sequence homology, the PETs show a similar overall structure: they are kidney-shaped, tightly packed molecules with a complex tertiary, intertwined folding and are composed of two domains, both containing β-sheets and α-helical structures (Fig. 1). An interdomain groove is the TCR-binding site; distinct MHC-II-binding sites have been identified. Each toxin has individual surface features explaining the individual mechanism of action and the low or absent serological cross-reactivity among toxins.

All SEs of the SE A group bind a Zn^{2+} ion by two histidines and an aspartate residue (in the case of SE A, residues His187, His225 and Asp227). This binding of Zn^{2+} is required for binding to MHC-II molecules (FRASER et al. 1992; ABRAHMSEN et al. 1995; HUDSON et al. 1995). A similar Zn^{2+}-binding motif

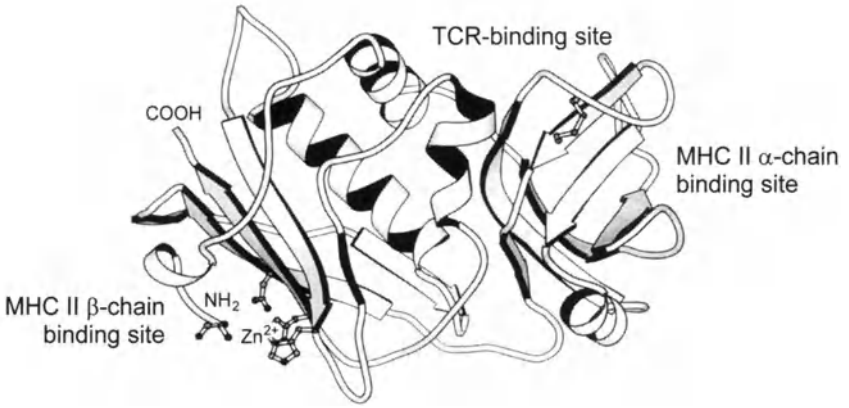

Fig. 1. Polypeptide fold of the staphylococcal enterotoxin A molecule (SE A). The binding sites for the variable part of the T-cell-receptor β chain, for the major histocompatibility complex (MHC)-II α chain and the Zn^{2+}-dependent binding site for the MHC II β chain are indicated. The drawing was made according to the published three-dimensional structure of SE A (Schad et al. 1995)

is present in SE C but not in SE B, but it is apparently not related to functional activity (BOHACH 1997).

II. Molecular Mechanism of Action

1. Binding to MHC Class-II Molecules

A hallmark of superantigens is their requirement for MHC class-II molecules on presenting cells to optimally stimulate T cells (FLEISCHER and SCHREZENMEIER 1988), as reflected by their binding to MHC class-II molecules (FLEISCHER et al. 1989; FISCHER et al. 1989; FRASER 1989; SCHOLL et al. 1989). Binding differs from normal presentation of processed peptide antigen, since superantigens bind as unprocessed proteins outside the antigen-binding groove of the class-II molecule (JARDETZKY et al. 1994; KIM et al. 1994). Fixed MHC-II-positive cells present superantigens effectively to T cells (FLEISCHER and SCHREZENMEIER 1988). However, although this basic mechanism is shared by all superantigens, individual members of the PET family have developed unique modes of interaction with the class-II molecule. Nearly all PETs (and superantigens in general) bind best – and some times exclusively – to human HLA-DR molecules (mouse H2-IE molecules) and less well to other isotypes of class-II molecules.

The crystal structures of the complex between HLA-DR1 and SE B or TSST-1 show that SE B and TSST-1 bind to overlapping regions on the monomorphic α-chain. Both toxins use residues of the N-terminal domain for this interaction. SE B binds at the side of the molecule, leaving the peptide-

binding site and the peptide exposed (JARDETZKY et al. 1994). This binding is of low affinity, and it has been suggested that, in the tri-molecular complex between SEB, class-II molecules and TCR, the TCR will interact with the class-II molecule to stabilize the complex (SETH et al. 1994). Evidence for an interaction between TCR and class-II molecules has been reported (FLEISCHER and MITTRÜCKER 1991; WEN et al. 1995).

TSST-1, in contrast, binds on the top of the α-chain and covers the α-chain α-helix peptide-binding site (KIM et al. 1994). Binding to the class-II molecule does not change the conformation of TSST-1 (MITCHELL et al. 1997). Although the binding sites of SE B and TSST-1 overlap on the class-II molecule, the two toxins do not compete for binding. This is due to the fact that the peptide within the antigen-binding groove influences the binding of TSST-1 (VON BONIN et al. 1995; WEN et al. 1996). TSST-1 binds only to a subset of class-II molecules containing the appropriate peptides; SE B does not bind to these class-II molecules. Thus, with TSST-1 (and probably other superantigens that use the same binding mechanism), *S. aureus* exploits the extreme variability of MHC-bound peptides to influence binding of its superantigenic toxins.

Members of the SE A group of PET have, in addition to the α-chain binding site, another binding site at the polymorphic β-chain of the class-II molecule involving the Zn^{2+} binding site. This binding is of high affinity and uses a bridge between the Zn^{2+} bound to SE A (via His187, His225 and Asp227) and His81 of the MHC class-II β-chain (ABRAHMSEN et al. 1995; HUDSON et al. 1995). Details of this interaction are described in a recent review (SVENSSON et al. 1997). This mechanism is also used by SEs D, E and I. Thus, SE A is able to bind (via two distinct binding sites) to two different sites on the class-II molecule and is, as a consequence, able to cross-link class-II molecules on the surface of the presenting cell by its own in the absence of a T cell. It has been shown that this cross-linking leads to transduction of signals in the cell, resulting in cytokine production in monocytes or co-stimulation of B cells (MOURAD et al. 1990; FULEIHAN et al. 1991). SE A competes with SE B and TSST-1 for binding.

Some PETs have the ability to form homodimers; in this way, they form functionally divalent molecules able to cross-link class-II molecules. The streptococcal SPE C forms a non-covalent homodimer that binds only to the β chain of the class-II molecule (by a Zn^{2+}-mediated binding). The low-affinity α-chain-binding sites of SPE C are covered by the dimerization (ROUSSEL et al. 1997; LI et al. 1998). SE D has been reported to form a Zn-dependent homodimer (AL-DACCAK et al. 1998). A covalent dimerization is achieved by SPE A, which has, in addition to the two cysteines that form the central disulfide loop, a third cysteine (FAGIN et al. 1998).

2. Non-MHC Receptors

Under certain conditions, SEs have also been shown to activate T cells in the absence of class-II molecules on accessory or target cells. Cytotoxic T cells can,

e.g., lyse target cells that do not express class-II molecules; however, much higher concentrations of SE are required (Dohlsten et al. 1991; Herrmann et al. 1991). This is not surprising, given the fact that PET can interact directly with the TCR (see below); therefore, a superantigen bound to any surface can, in principle, activate T cells. Whether there exists a second specific receptor or whether the SEs non-specifically stick to the surfaces of certain cells is unclear. Additional receptors have also been postulated, e.g., on endothelial cells or on thrombocytes, but have not been characterized (Fleischer and Hartwig 1992).

3. Interaction with the TCR

The major interaction site on the $\alpha\beta$ TCR is the variable part of the β-chain (Vβ; Table 3). Stimulation of murine or human T cells with a given toxin in vitro leads to selective expansion of $CD4^+$ and $CD8^+$T cells carrying certain Vβs (Janeway et al. 1989; Kappler et al. 1989; White et al. 1989). A similar preference (for Vγ) was reported for the response of $\gamma\delta$ T cells. Transfer of a given Vβ or of an epitope of hypervariable region (HV) 4 of the Vβ transfers

Table 3. Preferential stimulation of human T cells by bacterial toxins. Data are compiled from the literature. Note that, in most cases, determinations have been made with native and not with recombinant proteins; therefore, Vβ [variable part of T cell receptor (TCR) β-chain] specificities of staphylococcal enterotoxin (SE) and streptococcal pyrogenic exotoxin (SPE) may be affected by contamination with other toxins. The stimulation of human $V\beta2^+$ and $V\beta8^+$ T cells reported for native SPE A (Abe et al. 1991) and of $V\beta8^+$ T cells reported for native SPE C (Tomai et al. 1992) are not found with recombinant toxins (Braun et al. 1993)

Toxin	Reported preferences for T cells expressing Vβ of	
	Human T cells	Mouse T cells
SE A	1.1, 5.3, 6.3, 6.4, 6.9, 7.3, 7.4, 9.1, 18	1, 3, 10, 11, 12, 17
SE B	3, 12, 13.1, 13.2, 14, 15, 17, 20	3, 7, 8.1, 8.2, 8.3, 11, 17
SE C1	3, 12, 13.1, 13.2, 14, 15, 17, 20	3, 6.4, 12, 15
SE C2	12, 13.1, 13.2, 14, 15, 17, 20	3, 8.2, 10, 17
SE C3	12, 13.2, 14, 15, 17, 20	3, 5, 12, 13.2
SE D	5, 12	3, 8.2, 8.3, 11, 17
SE E	5, 6, 8, 18	11, 15, 17
TSST-1	2	3, 4, 15, 17
SPE A	12, 14, 15	8.2
SPE C	1, 2, 5.1, 10	
SMEZ	2, 8	n.a.
SSA	1, 3, (5.2),15	n.a.
YPMa	3, 9, 13.1, 13.2	7, 8.1, 8.2, 8.3
YPMb	3, 9, 13.1, 13.2	n.a.
MAS	3.1, 5, 7, 8, 10, 11.1, 12.1, 14, 17.1, 20	5.1, 6, 8.1, 8.2, 8.3

MAS, *Mycoplasma arthritidis* superantigen; *n.a.*, not available; *SMEZ*, streptococcal mitogenic exotoxin Z; *SSA*, streptococcal superantigen; *TSST*, toxic-shock-syndrome toxin; *YPM*, *Yersinia pseudotuberculosis* mitogen.

the specific response of the T cell to the recipient T cell (Choi et al. 1990; Fleischer et al. 1996). HV4 is not involved in peptide-antigen recognition and is located on an exposed lateral region. It is important to note that different Vβs bind to different toxins with different affinities; therefore, T cells with the highest affinity for a given toxin are preferentially expanded in bulk culture. This notion explains how T cells with certain Vβs can respond to toxins that are not preferentially expanded in such bulk cultures (Fleischer et al. 1991, 1996).

From structural and mutational data, a model of the ternary complex of SE, TCR and a MHC class-II molecule was hypothesized. The complementarity-determining regions (CDRs) of the TCR are oriented over the MHC peptide-binding site, with the Vβ domain bound to SEB and the Va domain above the class-II β1 domain (Jardetzky et al. 1994). This model is consistent with the findings that the TCR α-chain and the polymorphic MHC allotype influence the response of the T cell to superantigens. Although this model stresses the close similarity between T cell activation by antigen recognition and superantigens, there are some subtle molecular differences.

More recently, a soluble TCR Vβ-chain has been used to analyze the binding of PET. The soluble β-chain had the same specificity for different PETs as the T cell from which the β-chain was derived. Affinities ranged from 0.9μM to 140μM, similar to what had been found for the affinities of the TCR to specific peptide/MHC complexes (Li et al. 1998). The three-dimensional structure of the complex between a soluble TCR β-chain and SE C3 was determined. CDR2 of the β-chain and, to lesser extents, CDR1 and HV4, bind in a cleft between the small and large domains of the SE (Fields et al. 1996).

In spite of the requirement for class-II molecules in T cell stimulation, the toxins can also stimulate T cells in the absence of class-II molecules. SE-mediated cytotoxicity against several class-II-negative target cells has been found (Dohlsten et al. 1991, Herrmann et al. 1991). SE A covalently linked to antibodies against any cell-surface molecule can direct lysis of cytotoxic cells to class-II-negative target cells (Dohlsten et al. 1991), and toxins bound to plastic can, under certain conditions, activate T cells (Fleischer et al. 1991).

The molecular mechanism of T cell stimulation is thus a multivalent cross-linking of TCR molecules with MHC class-II molecules on the presenting cell. This cross-linking of the TCR, however, requires – at least at low toxin concentrations – additional adhesion molecules, such as CD2, CD11a or CD28. The CD4 or CD8 molecules do not appear to be required although, in some cases, CD4 interaction augments the response (Sekaly et al. 1991). By using this molecular mechanism, the superantigen uses the most efficient way to stimulate T lymphocytes. The use of class-II molecules as specific receptors restricts the T cell interaction to the professional APCs, most likely the dendritic cells, since macrophages are dispensable for T cell activation in vivo (Bette et al. 1993).

III. Biological Significance of PETs

1. Role of PETs as Virulence Factors

Although superantigens act on T cells of different species, it appears that they work best in the species that is a natural host for the producing pathogen. The toxins derived from *S. aureus* and *S. pyogenes* (bacteria not found in wild mice) stimulate human T cells much more efficiently than murine T cells, whereas the opposite is true for the mitogen derived from *M. arthritidis* (a natural pathogen for rodents; FLEISCHER et al. 1991). TSST-1 or SE C produced by ovine strains of *S. aureus* is much more efficient on ovine T cells than on human T cells (LEE et al. 1992; MARR et al. 1993). This suggests that these molecules have been adapted (via evolution) to the MHC and TCR molecules of the natural host. Acquisition by gene transfer of toxin genes and their subsequent mutation, such as transfer of *tst* genes from human to ovine and bovine *S. aureus* strains, may be a mechanism of such adaptation.

For several reasons, it may be expected that the production of a superantigen offers an evolutionary advantage for the respective pathogen. First, the molecular mechanism of action (the cross-linking of TCR Vβ with class-II molecules) has separately evolved several times. Second, the extensive genetic polymorphism of the PET toxins argues that the presence of multiple forms of a superantigen is advantageous for the producing species. Finally, it appears that superantigens have been adapted to the host's immune system by the producing pathogen, because they have the highest mitogenic potential in the immune system of their respective natural host.

Thus, the evolutionary advantage can be expected to be directly or indirectly linked to the T-cell-stimulatory function of superantigens. This has indeed recently been shown for the mouse mammary-tumor viruses; T lymphocyte stimulation is required for effective multiplication and transmission of the virus (HELD et al. 1994). For the gram-positive cocci, there is no clear evidence of an advantage of T cell stimulation. The decisive mechanisms against these extracellular bacteria are granulocytes, antibodies and complement. It is, however, noteworthy that production of a superantigen leads to delayed clearance of *S. aureus* in infected mice (ROTT and FLEISCHER 1994). Moreover, it has been repeatedly shown that humoral and cellular immune responses are diminished if a SE is injected along with a conventional antigen (WEN et al. 1997).

2. Role in Pathogenicity

Given the extremely low concentration (in pg/ml) required for T cell stimulation and the large amount of toxin secreted into the culture medium by some strains of staphylococci and streptococci, it is clear that a small focus of a bacterial infection can be sufficient to induce a general activation of the immune system and a massive expansion of responding T cells. In patients with toxic shock due to TSST-1-producing *S. aureus*, V$\beta 2^+$ cells expand to up to 70% of

all peripheral blood T cells (Choi et al. 1990). Consequences of such polyclonal T cell stimulation can be shock, immunosuppression and autoimmunity.

The shock-like symptoms induced by these toxins are caused by a massive release of lymphokines (tumor necrosis factor-α, interferon-γ, interleukin-2) and monokines (interleukin-12). Similar symptoms are observed after the first injection of stimulating anti-TCR antibodies in patients requiring immunosuppressive therapy. Critical mediators in this shock-like symptom are apparently tumor necrosis factor-α and interferon-γ, both are produced by T lymphocytes (Bette et al. 1993). The same mediators are involved in the septic shock induced by endotoxins of Gram-negative bacteria. It appears that, while distinct cell populations are stimulated in Gram-positive and Gram-negative shock, in both cases, a similar cytokine cascade is initiated that ultimately leads to the same terminal events. Toxic-shock syndrome is observed during human infections with *S. aureus* and *S. pyogenes*. It is interesting to note that not all SEs have been implicated in such diseases. So far, TSST-1 and SE B seem to be almost exclusively involved in staphylococcal toxic shock. Although the gene for TSST-1 is widely distributed among staphylococci, a single *S. aureus* clone accounts for the majority of toxic-shock cases in five countries (Musser et al. 1990). This indicates that not the superantigen alone but rather a combination of virulence factors determines pathogenicity. A greater heterogeneity was found with *S. pyogenes* isolates from patients with streptococcal toxic-shock-like syndrome. Here, the ability to cause this syndrome is associated with all of the known SPEs, but SPE A-producing bacteria cause a high proportion of cases. Interestingly, two of four *speA* alleles were present in the single-bacterial clones that caused the great majority of these cases (Nelson et al. 1991).

Immunosuppression can result, because stimulation of many T cells will impede the coordinate immune response. Moreover, a toxin can induce anergy and death by apoptosis in those T cells responding to it. After injection of an SE into mice, the initially responding T cells are partially deleted, and those remaining that are still present in the spleen do not respond to SE B or to anti-T-cell-receptor antibodies. It is also conceivable that activated $CD8^+$ T cells could destroy APCs or B lymphocytes that have toxin molecules bound to their class-II antigens.

3. Association with Human Autoimmune Disease

The polyclonal activation of T cells by PET could bypass the control of autoreactive T cells, or helper T cells could be focused on autoreactive B cells and induce autoantibody production. This may be of relevance, e.g. in streptococcal diseases that are associated with autoimmune phenomena. Stimulation of autoreactive B cells by cross-reactive epitopes (of, e.g., M proteins) and the generation of help from T cells stimulated by erythrogenic toxins may provide a pathogenetic mechanism. The requirement for such an interaction of two different pathogenicity factors of group-A streptococci could explain why such

post-streptococcal autoimmune diseases are relatively rarely found. In experimental models, it was shown that control of autoreactive T cells could be bypassed by injection of a superantigen (Schiffenbauer et al. 1998). An ongoing autoimmune reaction against brain or joint antigens was aggravated by the injection of a superantigen (Cole and Griffiths 1993; Schiffenbauer et al 1998). A resolved experimental allergic encephalomyelitis could be exacerbated by injection of a superantigen stimulating the appropriate autoreactive T cells. Moreover, in naive animals, polyclonal T cell activation by a superantigen can prime Vβ-specific T cell responses to a self antigen by a non-Vβ-specific mechanism of "innocent-bystander" activation (Rott et al. 1995).

Several groups have provided indirect evidence of the involvement of superantigens in human diseases. In all these cases, an imbalance in the Vβ composition of T cells from patients has been found. Two groups have demonstrated a selective increase in T cells carrying Vβ-14 in the synovial fluid of patients with rheumatoid arthritis (Kotzin et al. 1993). Furthermore, an imbalance in the Vβ repertoire of T cells has repeatedly been reported in patients with acquired immune deficiency syndrome, although this has not been confirmed by all investigators. It is still unclear, however, if this is due to a superantigen produced by human immunodeficiency virus or to secondary infection or to a non-superantigen-dependent mechanism. While the idea that superantigens are at work in such diseases is very exciting, one has to keep in mind that preferential stimulation of certain Vβ-bearing T cells can also occur during a normal immune response (Boitel et al. 1992). The findings of an increase in Vβ2$^+$ and Vβ8$^+$ T cells in Kawasaki syndrome, an acute multisystem vasculitis in children, are interesting (Abe et al. 1992). Staphylococci and streptococci producing PETs have been isolated from these patients; their pathogenic relevance, however, is still unclear.

In patients with atopic dermatitis, a chronic inflammatory skin disease associated with pruritus and elevated serum immunoglobulin E, persistent colonization with *S. aureus* is a well-known feature. There is evidence that SEs produced by the staphylococci have the potential to trigger chronic T-cell-mediated skin inflammation (Herz et al. 1998). It is also a common observation that exacerbations of psoriasis are often associated with streptococcal infections, and a causal link has been suggested (Ortonne 1996).

4. The Enterotoxic Activity

The SEs are named for their ability to induce a gastrointestinal illness within 2–4 h upon oral uptake of a few micrograms in primates. Studies with mutant molecules (Hufnagle et al. 1991, Harris et al. 1993) and with chemically modified SEs (Alber et al. 1990) indicate that the enteropathogenicity of SE is not caused by action on T cells. It has been suggested that the symptoms of gastrointestinal intoxication are caused by leukotrienes and histamine released from mast cells in response to substance P in the mucosa (Alber et al. 1989). The receptors involved are still obscure.

D. Other Superantigens (or Pseudosuperantigens) of Gram-Positive Cocci?

In addition to the PEs, there are other staphylococcal and streptococcal proteins (genetically unrelated to the enterotoxins) that have been proposed to be superantigens, such as streptococcal M protein, streptococcal erythrogenic toxin B and staphylococcal epidermolytic toxins (ETs). However, the evidence discussed below suggests that these molecules are not superantigens; their mitogenicity is due to artifacts (Fleischer et al. 1995).

I. The ETs of *S. Aureus*

ETs A and B of *S. aureus* share a high sequence homology but are serologically unrelated. They are able to dissociate intercellular adhesion molecules in the skin, leading to subgranular epidermolysis. ETA is synthesized as a 31-kDa precursor molecule that is subsequently cleaved into a mature polypeptide of 27 kDa. In the context of this review, it is noteworthy that ETs A and B have an intrinsic esterase activity and are homologous to the staphylococcus V8 protease (Bailey and Redpath 1992). They share the catalytic triad at the proteinase active center. Mutation of the serine of the active center leads to loss of epidermolytic activity in mice. ETA was reported to be a superantigen stimulating Vβ2$^+$ T cells (Marrack and Kappler 1990). Initially, all work with ETA used a preparation available from Toxin Technology Inc., Sarasota, Florida. At least 1–10 μg/ml of this preparation were required to obtain maximal responses, in contrast to the few nanograms required for SE. It has been shown that ETA does not bind MHC-II molecules (Herrmann et al. 1989). Recombinant ETA was produced in *S. aureus* and assayed for mitogenic activity towards human peripheral blood mononuclear cells (PBMsC) or Vβ2$^+$ T cells, but no mitogenic activity could be detected, whereas the preparation from Toxin Technology was stimulatory for PBMC and Vβ2$^+$ T cells (Fleischer and Bailey 1991). Although this issue is still controversial (Iandolo and Chapes 1997), all these findings support the notion that ETs are not superantigens. To reconcile these findings, it has recently been proposed that ETs may be atypical superantigens (Monday et al. 1999).

II. M Proteins and SPE B of *S. Pyogenes*

M proteins of *S. pyogenes* are major virulence factors of group-A streptococci. They are receptor proteins that show multiple binding to different plasma proteins and protect streptococci against phagocytosis. They form a family of closely related proteins protruding from the cell surface. The variable N-terminal portion of the molecule carries the M-type specific-antigen epitopes. M-protein fragments produced by limited proteolytic digestion of whole streptococci with a molecular mass of 33 kDa (pepM5) have been proposed to be

superantigens and to stimulate Vβ2$^+$, -4$^+$ and -8$^+$ human T cells (Tomai et al. 1991, 1992).

If, however, M5-protein or pepM5 are thoroughly purified, they are no longer mitogenic (Fleischer et al. 1992; Esaki et al. 1994; Degnan et al. 1997). The stimulatory activities for Vβ2$^+$ and Vβ8$^+$ T cells could be separated from each other, suggesting that the superantigenic activity of M-protein type 5 could be due to contamination with at least two different, possibly novel SPEs (Schmidt et al. 1995). Class-II binding by pepM5 has not been observed by other investigators (Herrmann and MacDonald, personal communication). It has recently been reported that even recombinant M5 fragments are mitogenic for T cells (Watanabe-Ohnishi et al. 1994). This finding has not yet been confirmed, but other groups have not been able to find mitogenicity with recombinant M5 protein or its fragments (Robinson et al. 1991; Schmidt et al. 1995; Degnan et al. 1997).

Taken together, the available evidence strongly suggests that mitogenicity of M-protein preparations is not due to an intrinsic activity but to contaminating SPEs, some of them possibly novel or unknown at the time of the reports. The permanent detection of novel SPEs invalidates the suggestive argument that the Vβ pattern of T cells that are stimulated by M proteins does not match that of any of the known SEs or SPEs (Tomai et al. 1992). The reported reactivity of pepM with human cells but not mouse cells can be explained. At low concentrations, most SEs and SPEs, similar to pepM, show a preference for human as opposed to mouse cells, because most SEs and SPEs bind much better to human class-II molecules. All data about the superantigenicity of M protein are derived from the preparations of a single laboratory and still await confirmation.

The same situation was found with SPE B, which is identical to the precursor of the streptococcal proteinase. It is a molecule of 371 residues, with a molecular weight of 40 kDa, and is processed to a mature protease of 253 amino acids and 27 kDa. All group-A streptococci have SPE B and, in the genome, there is only one non-polymorphic gene for this molecule. SPE B has no homology to any member of the SE family (Betley et al. 1992). SPE B has been reported to selectively stimulate Vβ8$^+$ human T cells. Upon proper purification of SPE B, however, it became evident that the superantigenic properties of these molecules are due to contamination with a novel, Vβ8-stimulating PET (Braun et al. 1993), and recombinant SPE B was not mitogenic (Gerlach et al. 1994). The Vβ8-stimulator [designated SPE X or Streptococcal mitogenic exotoxin Z (SMEZ)] has recently been isolated and cloned (Kamezawa et al. 1997; Gerlach et al. 1999; Proft et al. 1999). Four different variants have been found.

III. The Mitogenic Factor of *S. Pyogenes*

A protein of 26 kDa, the so-called mitogenic factor (MF), recently designated SPE F (Norrby-Teglund et al. 1994), was purified from *S. pyogenes* strain NY5 (the prototype strain from which SPEs A–C and SMEZ were originally

purified). It was subsequently cloned, and the recombinant protein displayed stimulatory activity on rabbit PBMCs (Iwasaki et al. 1993). The recombinant protein was not tested on human T cells. We have not been able to detect mitogenicity with recombinant MF (Reichardt et al., unpublished). MF is not polymorphic and is elaborated by all group-A streptococci but not by other streptococci. Interestingly, it has enzymatic activity; it is a nuclease (Iwasaki et al. 1997) and is identical to DNase B (Reichardt et al., unpublished). The Vβ profile of the stimulated human T cells was recently described to consist of Vβ2, -4, -8, -15 and -19, surprisingly similar to that of pepM5 (Norrby-Teglund et al. 1994). This could indicate either that the superantigen activity of pepM5 is due to MF or – more likely – that both pepM5 and MF are contaminated by the same novel SPEs. It is interesting that, in this context, antibodies against recombinant SPE X/SMEZ specifically block the mitogenic activity of MF (Gerlach et al. 1999).

Taken together, these results raise an important caveat for work with T cell mitogens from *S. aureus* and *S. pyogenes*. Because SE and SPE are active in concentrations of a few picograms per milliliter, the sensitivity of T cells to these stimulators is several orders of magnitude higher than the sensitivity of any biochemical or serological test, and contamination with an SE or SPE is extremely difficult to exclude. Such contamination has been shown to occur even if a recombinant protein is purified on columns previously used for purification of natural enterotoxin. Moreover, given the high polymorphism and unequal distribution of the PET, it is surprising that non-polymorphic proteins like SPE B or MF, which are present in all *S. pyogenes* isolates, are superantigens. A number of different proteins of *S. pyogenes* have been purified from culture supernatants and have been found to behave like superantigens (Fleischer et al. 1995). The possibility that *S. pyogenes* has evolved several unrelated superantigens is exciting, but we should await confirmation of their activity using recombinant proteins.

The surprising finding that the unique molecular mechanism of T cell stimulation has been developed completely independently by infectious agents of four different classes (gram-positive cocci, gram-negative bacteria, a *Mycoplasma* and retroviruses) has lead to the expectation that additional superantigens produced by other infectious pathogens will be found. This expectation, together with the appealing designation "superantigens", has induced a boom in reports of novel superantigens. However, the number of reported candidates far exceeds the number of superantigens that is likely to exist. Even a lectin has been reported to act as a superantigen (Galleli et al. 1993), but this has not been confirmed yet. For several candidates, it is already now reported that attempts to confirm the initial claim have failed or have prompted contradiction. We have not been able to find any superantigenic activity either with purified enterotoxins of *Clostridium perfringens* (both purified from *C. perfringens* cultures or as a recombinant molecule from *Escherichia coli*; Krakauer et al. 1997) or with the exotoxin A of *Pseudomonas aeruginosa* (unpublished observations).

E. The *M. Arthritidis* Superantigen

M. arthritidis is a pathogen of rodents and induces an acute inflammatory infection in rats and mice. In mice, this inflammation is followed by a chronic joint disease. Cell-free supernatants of *M. arthritidis* contain a potent T cell mitogen acting on T cells of several species. Although this mitogenic principle has been known for more than 15 years (Cole and Atkins 1991), the protein was cloned only recently (Cole et al. 1996). This is due to the small amounts of *M. arthritidis* superantigen (MAS) in the *Mycoplasma* culture and to the lability and adhesive property of MAS. MAS is a hydrophobic, basic peptide that is heat-labile at 56°C and susceptible to serine proteases. The sequence reveals a protein unrelated to any other superantigen, with 153 amino acids, a calculated molecular mass of 25 kDa and a very basic pI of 10 (Cole et al. 1996).

MAS has all the functional properties of superantigens (Matthes et al. 1988; Cole et al. 1990). Its T cell-stimulatory properties are strictly dependent on the presence of H2-IE molecules or HLA-DR molecules on the stimulating cells. MAS stimulates both $CD4^+$ and $CD8^+$ T lymphocytes, and responsive T cells have been described. There is a preferential stimulation of mouse T cells expressing Vβ6, -8.1, -8.2 and -8.3, and of human T lymphocytes expressing Vβ17.1 (also designated 19.1; Table 3). In addition, there are other TCRs that respond to MAS, but with lower affinity (Fleischer et al. 1991).

Injection of MAS into experimental animals induces a mild toxic-shock syndrome and pronounced T cell suppression (Cole and Wells 1990). This is only found in MAS-responsive mouse strains that posses H2-IE molecules. The chronic joint disease is only induced by infection with the *Mycoplasma* itself or by intra-articular injection of MAS. The role of the superantigen in the induction of arthritic disease is still not clear. It is noteworthy that MAS injection induces a flare-up in mice that have recovered from collagen-induced arthritis. It has been noted that some parameters of the *M. arthritidis*-induced disease can also be found in rheumatoid arthritis and that antibodies to MAS are present in some patients with this disease. An involvement of MAS-like superantigens in the pathophysiology of rheumatoid arthritis has been suspected (Knudtson et al. 1997).

F. The *Y. Pseudotuberculosis* Mitogen

The three pathogenic species of *Yersinia* (*Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis*) produce many virulence factors required for survival in the body. *Y. enterocolitica* and *Y. pseudotuberculosis* have been reported to produce superantigens. Whereas the putative superantigen of *Y. enterocolitica* still awaits isolation and confirmation, a superantigen designated *Y. pseudotuberculosis* mitogen (YPM) has been purified and cloned (Ito et al. 1995; Miyoshi-Akiyama et al. 1995). YPM is synthesized as a 151-amino-acid

precursor that is cleaved to a 14-kDa, 131-amino-acid mature protein. It has no homology with any other superantigen. The protein has all the characteristics of a superantigen in vitro and in vivo. It is produced (with distinct geographical distribution) by certain serotypes of *Y. pseudotuberculosis*. The gene encoding YPM was detected in 95% of clinical isolates of *Y. pseudotuberculosis* from Japan or Far East Russia but only in 17% of European clinical isolates (Yoshino et al. 1995). Recently, a variant YPM was isolated and cloned (Ramamurthy et al. 1997). The two YPMs (designated YPMa and YPMb) have 83% homology. This interesting finding indicates that YPM forms a polymorphic family as do SEs.

The infection with *Y. pseudotuberculosis* is characterized by acute mesenteric inflammation, lymphadenopathy and systemic symptoms, such as fever and leukocytosis. In patients with *Y. pseudotuberculosis* infections, an elevation of Vβ3-bearing T cells has been found (Abe et al. 1997). Several immunopathological diseases, such as reactive arthritis or uveitis and erythema nodosum, can follow an infection with *Y. pseudotuberculosis*. Moreover, Kawasaki syndrome has been linked to *Y. pseudotuberculosis* infections. A role of the superantigen YPM in the pathology of this infection is conceivable but is still not clear.

G. Concluding Remarks

The toxins described in this chapter constitute the most efficient T-lymphocyte stimulators known. Although their exact roles as virulence factors are not established, it is obvious that their induction of immunosuppression, destruction of APCs and T cell anergy should be of advantage for the infecting microorganisms. The manifold mechanisms of interaction with variable target structures of the immune system, the MHC-II molecule and the TCR allow a maximum of biological efficacy combined with a minimum of immunological cross-reactivity. This is the basis for the extensive polymorphism of the toxins, which provides the producing species with a multitude of non-cross-reacting toxins. The polymorphism is probably maintained and extended by permanent modifications of the toxin genes through mutations and selection for toxin variants that are optimally stimulatory for the host's immune system.

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CHAPTER 27

Structure and Activity of Endotoxins

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A. Introduction

Gram-negative bacteria comprise a great number of pathogenic microorganisms causing a variety of diseases in mammals, including man. Many of the clinical symptoms observed following an infection with gram-negative pathogens are specific for the infectious species; however, some symptoms are commonly observed and are thus species independent (Brade et al. 1988). These symptoms, such as fever, were attributed to the action of heat-stable toxins that were recognized to be associated with the bacterial cell and were therefore termed endotoxins (Wolff 1904; Westphal and Lüderitz 1954; Rietschel and Brade 1992) to distinguish this class of toxins from actively secreted exotoxins, which are proteins and are thus heat labile (Bhakdi et al. 1994). The chemical characterization of endotoxins revealed that they are composed of a lipid component and a carbohydrate component; thus, they are lipopolysaccharides (LPSs) because of their chemical nature (Westphal and Lüderitz 1954).

The lipid that anchors LPS to the outer membranes of gram-negative bacteria can be released chemically by mild acid treatment and was termed lipid A. In order to identify the toxic principle of LPS (in analogy to the discovery of toxophore groups in some exotoxins), biological activities of lipid A and the released polysaccharide were investigated. Since the polysaccharide moiety was not toxic (Westphal and Lüderitz 1954), it was assumed that toxicity was associated with lipid A. Whereas some groups observed adjuvant activity, tumor necrosis and fever after intravenous injection of lipid A, others did not observe these effects (Galanos et al. 1972). Final evidence came from complexation of lipid A with albumin which, by increasing its solubility, eliminated the observed discrepancies and proved its bioactivity (Galanos et al. 1972). To further identify the molecular requirements enabling lipid A to elicit endotoxic effects, a vast number of natural and synthetic molecules were subsequently analyzed. Structure–activity studies led to the discovery of antagonistically active lipid A, which served as lead structures for the development of synthetic compounds (Qureshi et al. 1998). The isolation of LPS from different gram-negative bacteria, their chemical characterization and comparison of their biological activities revealed the structural requirements for their biological activity and showed that not all LPS are endotoxically active (Rietschel et al. 1996).

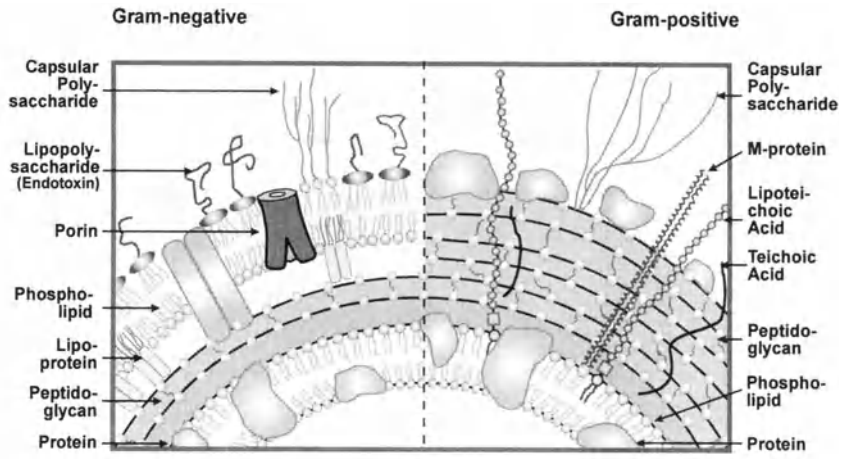


Fig. 1. Schematic representation of the bacterial cell wall. The cellular compartment of bacteria is always surrounded by a cytoplasmic membrane consisting of a phospholipid bilayer and proteins. This membrane is surrounded by a covalent meshwork of peptidoglycan (murein) which, in cases of gram-negative bacteria, possesses only a few layers. Peculiarly, this class of microorganisms has an outer membrane including a second cellular compartment (periplasmic space). Lipopolysaccharides form the outer leaflet of the outer membrane. Porins [outer-membrane protein (Omp)F/C] mediate the selective uptake of hydrophilic nutrients, and structural proteins (OmpA, lipoprotein) contribute to the membrane's stability and its linkages to peptidoglycan. Capsular antigens are shown as examples of further glycosyl-based surface molecules. In contrast, gram-positive bacteria are surrounded by a thick murein multilayer. In addition, the cell walls of these microorganisms also consist of polysaccharides (lipoteichoic acid, teichoic acid, capsular polysaccharides) and/or proteins (partly also organized in complex surface layers)

The biological significance of LPS may be viewed from two perspectives. They are major components that constitute the outer leaflet of the outer membrane of most gram-negative bacteria (Fig. 1). LPS are thought to be indispensable for the viability of these bacteria since, with a single exception, mutations leading to loss of the ability to assemble functional LPS were conditional lethal (Rick and Osborn 1977; Belunis et al. 1995). This reflects the importance of LPS for the bacterial cell. Remarkably, very recently, a mutant strain of *Neisseria meningitidis* lacking the gene *lpxA* was constructed. This mutant was viable and appeared to be devoid of LPS, since outer-membrane complexes of the mutant did not contain 3-hydroxylated fatty acids, regarded as a marker for LPS. In addition, the reactivity with LPS-specific antibodies was lost and, on sodium dodecyl sulfate polyacrylamide-gel electrophoresis, no LPS was detected after silver staining (Steeghs et al. 1998). The development of the outer membrane as the outmost part of the cell envelope of gram-negative bacteria may be seen as an adaptation to allow survival in rapidly changing environments. The space created between the cytoplasmic membrane and the outer membrane, termed periplasm, allows the build-up of

concentration gradients over the cell membrane and represents a regulable environment suitable for the proper folding and activity of proteins, such as enzymes and binding proteins. At the same time, the outer membrane protects the vulnerable inner cell membrane, a phospholipid bilayer, from mechanical and chemical stress. Due to their chemical and physical properties, LPS represent an effective barrier for hydrophobic agents and protect the bacterial cell from the action of, e.g., bile salts and hydrophobic antibiotics. These properties are conferred mainly by the lipid part of the molecule. Due to its exposed location, the polysaccharide represents the main target of the humoral immune response and shields bacteria from attack by the immune system of the host, thereby maintaining the integrity of the membrane. For pathogenic enterobacteria, it is known that the most exposed polysaccharide region of LPS inhibits the phagocytosis of microorganisms by macrophages and may offer protection against the lytic effect of the serum complement system. LPS are found only in gram-negative bacteria and, because of their importance for the viability of the bacteria, LPS and its biosynthesis have been identified as promising targets for the development of new classes of antimicrobial drugs.

However, some LPS were recognized as highly active toxins in mammals (Rietschel and Brade 1992). The toxic effects, such as high fever, hypotension, leukopenia, tachycardia, tachypnea, systemic intravascular coagulation accompanied by acute-respiratory-distress syndrome, and multi-organ failure in final stages (known as manifestations of bacterial sepsis; Vincent 1996), were first believed to be the result of the destructive effects of the invading microorganism. The role of LPS in pathogenicity was in dispute for decades, because similar host responses were seen after infection with gram-positive microbes (Stegmayr et al. 1992) or even following events other than infection (Parillo 1993). Likewise, positive blood cultures (bacteremia) or endotoxin in the blood (endotoxemia) were not a prerequisite for the diagnosis of septic shock (Marchant et al. 1995). However, many of the biological effects seen during an infection can be elicited by LPS or natural or synthetic lipid A alone (Galanos et al. 1985), indicating that endotoxins are involved in the manifestation of septic shock. In fact, very recently, bacterial mutants that express non-toxic lipid A have been constructed (Nichols et al. 1997; Sunshine et al. 1997; Khan et al. 1998). These mutants multiply in vivo without causing lethality, thus proving that LPS has an important role in lethal infections.

LPS are released in small quantities during cell fission but in larger amounts during cell death, e.g., as a consequence of antibiotic treatment or complement-mediated lysis. Another source of LPS is bacteria of the gut, although LPS and bacteria do not penetrate the intact gut mucosa at high rates (Brearly et al. 1985). After invasive treatment or severe burns, larger amounts of LPS and/or bacteria may enter the bloodstream (Bahrami et al. 1996). Nevertheless, some strains of, e.g., *Escherichia coli*, *Shigella* and *Yersinia* are able to adhere to epithelial cells and penetrate the intact gut wall (Levine 1987). The main route by which endotoxins enter the blood from the gut leads

through the portal vein to the liver, where they may be taken up by Kupffer cells, released into the bloodstream and subsequently redistributed into hepatocytes, which finally secrete them together with bile (van Deventer et al. 1988; Freudenberg et al. 1992). Alternatively, endotoxins may enter the bloodstream via the thoracic duct, thereby circumventing the liver.

The most common cause of endotoxic shock is a generalized infection with a gram-negative pathogen that spreads into different organs. In former days, natural infection (the main source of endotoxins) occurred more frequently, due to lower hygienic standards and less knowledge of the sources and routes of infection. Identification of pathogens, the development of diagnostic tools, the discovery of antibiotics, their introduction into medicine and improvement of hygienic conditions have led to major improvements for the treatment and prevention of such infections. However, in terms of the absolute number of deaths, the mortality of severe infections with gram-negative pathogens remained almost unaltered. Nosocomially contracted infections are now the main cause of endotoxic shock, due to the introduction of invasive surgical treatment and mechanical ventilation in intensive-care units.

The importance of endogenously produced mediators for the induction of endotoxic symptoms has been clearly established. Typical endotoxic effects can be elicited by recombinant cytokines, such as tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β) and IL-6 (Vogel 1990). Additionally, endotoxic symptoms were correlated with serum levels of these mediators (Zabel et al. 1989), and antibodies against them were able to suppress the endotoxic activities of LPS (Beutler and Cerami 1988). The cellular source of these mediators has been identified by cell-transfer experiments from endotoxin-sensitive mice to resistant mice (Freudenberg et al. 1986). Although, in principal, a large number of different cell types is able to respond to LPS challenge, only transfer of macrophages (not of granulocytes or lymphocytes) could sensitize mice that are naturally resistant to LPS. Therefore, the key step involved in endotoxin action is the activation of monocytes/macrophages to produce cytokines, such as TNF-α and IL-1β, which themselves elicit pleiotropic biological effects (Fig. 2). The onset of septic shock depends on the development of a systemic, dysregulated inflammation reaction that involves multiple cellular and humoral events (Fig. 2).

The significance of endotoxins for the outcome of infections with gram-negative pathogens is difficult to assess due to the concerted action of different virulence factors and the different dispositions of patients. In infections that remain local, e.g., those often observed for *N. gonorrhoeae*, small amounts of released endotoxins lead to the activation of the host's immune response, attracting immune-competent cells to the site of infection and thereby promoting clearance of bacteria. However, systemic infection [as typically observed in typhoid fever caused by *Salmonella enterica* serovar (sv.) Typhimurium, or after spreading from locally infected sites to different organs] can lead, in severe cases, to organ failure and, ultimately, death. The biological role of endotoxins in infections with living bacteria has been addressed by

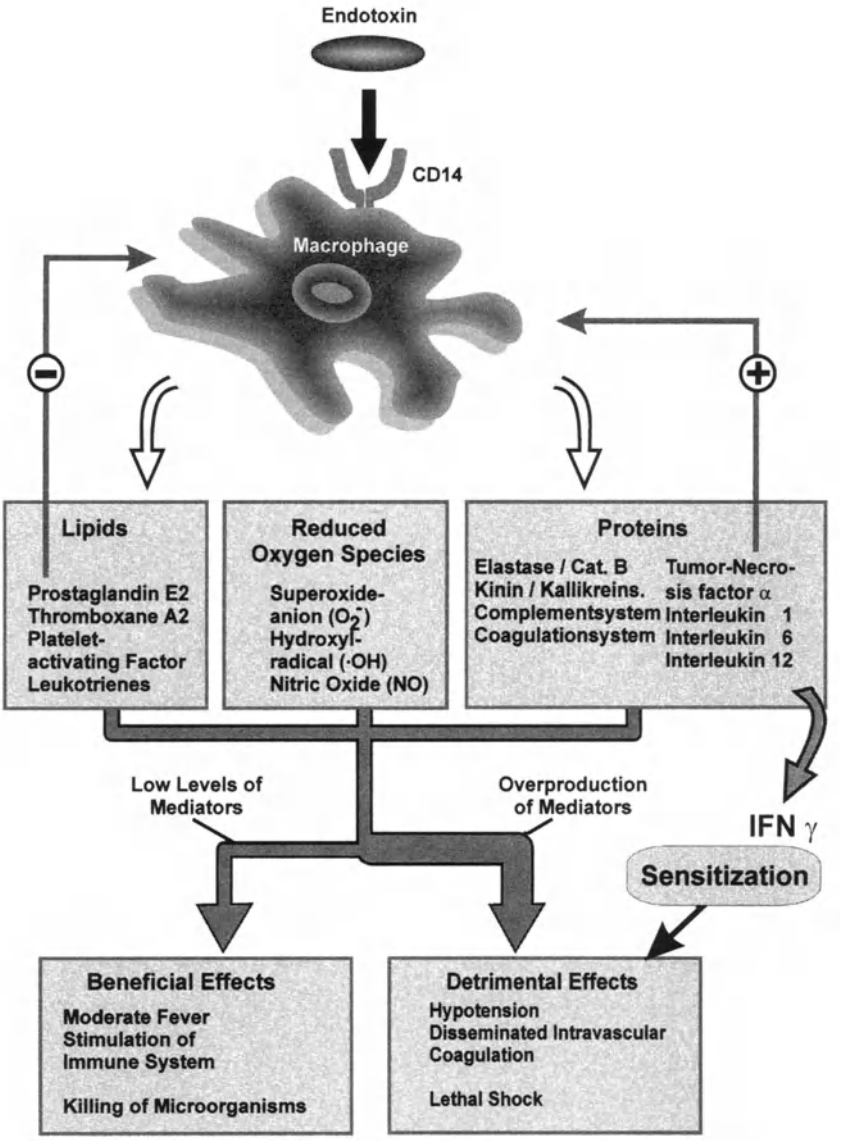


Fig. 2. Effector mechanism of endotoxins. Bacterial endotoxins activate macrophages and monocytes to produce and release endogenous mediator molecules and antimicrobial factors. These can be classified into three different classes of substances. Reduced oxygen species and enzymes are efficient poisons for bacterial pathogens. Cytokines, especially tumor necrosis factor α, are proteins that contribute to the enhancement of the inflammatory reaction, including the antimicrobial activity of phagocytes. Lipid mediators, such as prostaglandin E2 stemming from the metabolism of arachidonic acid, partially inhibit exuberant formation of mediators. An increased defense against infection is achieved by all these molecules when released in small quantities. In the presence of large amounts of lipopolysaccharide, however, noxious overproduction of mediator molecules is induced; this may lead to lethal shock through further reinforced cascade-like reactions. In the presence of low endotoxin concentrations, interferon γ also stimulates macrophages to react by overproducing mediator molecules and renders the host organism hyperreactive to endotoxin

the construction of genetically engineered bacterial strains of *S. enterica* sv. Typhimurium and *Haemophilus influenzae* that, when mutated in the *waaM* locus (previously termed *htrb*), synthesize a structurally altered lipid-A molecule with reduced endotoxicity (NICHOLS et al. 1997; SUNSHINE et al. 1997). In the case of the mutant *Salmonella* strain, the genetic defect led to severe limitation of its ability to colonize organs and to cause systemic disease (SUNSHINE et al. 1997). The importance of endotoxin for the development of organ damage has been shown by the construction of a mutant strain of *S. enterica* sv. Typhimurium unable to assemble an endotoxically active LPS but with unaltered ability to colonize and multiply within organs (KHAN et al. 1998). This mutant was cleared without causing death in experimentally infected mice.

As septic shock progresses, an irreversible state is reached in between a few hours and a few days. The shortness of the period following the first indications (such as chills and fever) demands the development of fast-acting drugs or preventive treatment. Despite decades of intensive research and a great demand for alternatives, conventional antibiotic treatment remains the main weapon used by clinicians in the treatment of such infections. As the toxic effects exerted by endotoxins are independent of the viability of the bacteria, and considering the increasing resistance of pathogenic bacteria to antibiotics, the search for alternative strategies is of major importance. The eradication of bacteria and suppression of the activation cascade at early steps appear to be the most promising approaches for successful treatment. The structural analysis of LPS (HOLST and BRADE 1992), the identification of their biosynthetic pathways (RAETZ 1996) and the identification of events that lead to activation of the immune response at a molecular level, i.e., the interaction of endotoxins with host target cells, will certainly provide a rational basis for the development of new antibacterial drugs.

In recent years, LPS from certain pathogens have been recognized to be associated with other diseases, such as chronic gastritis (MORAN and ASPINALL 1998) and the Guillain-Barre syndrome (GBS; PRENDERGAST et al. 1998), whose biological effects (the induction of pepsinogen production and release, and the induction of autoreactive antibodies, respectively) are independent of endotoxicity. Because of these examples, structural analysis of LPS remains an important tool to identify such relationships, creating the possibility of antibiotic treatment of diseases not previously thought to be related to bacterial infection.

B. The Chemical Structure of LPS

Due to differences in colony morphology, smooth (S) bacteria can be distinguished from rough (R) bacteria. These observed differences reflect differences in LPS structure (Fig. 3); therefore, S-type LPS is distinct from R-type LPS. Generally, LPS consist of a carbohydrate chain of varying length, which

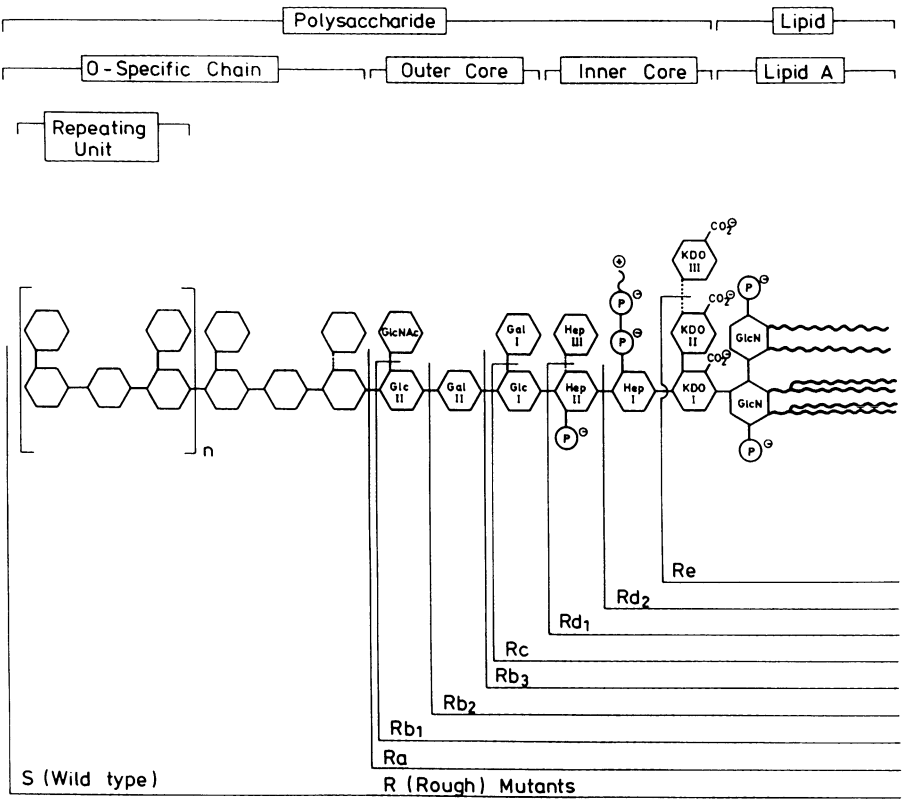


Fig. 3. Schematic representation of the structure of *Salmonella enterica*, wild type and rough-mutant lipopolysaccharides (LPS). According to chemical, biosynthetic, biological and genetic criteria, LPS can be divided into three regions: the O-specific chain, core oligosaccharide and lipid A. The O-specific chain represents a polymer of repeating units characteristic for each bacterial strain. The terms Ra–Re refer to structures of LPS from rough mutants that (due to genetic defects) synthesize a truncated core oligosaccharide and, therefore, lack an O-specific chain. The smallest LPS structure found in viable *Salmonella enterica* strains consists of lipid A and two 3-deoxy-D-*manno*-2-octulosonic acid (2-keto-3-deoxy-D-*manno*-octonic acid; Kdo) residues (Re-mutant). Saccharide groups are depicted by *hexagons*, and *dotted lines* mean non-stoichiometric substitutions. *Gal* D-galactose, *Glc* D-glucose, *GlcN* D-glucosamine, *GlcNAc* *N*-acetyl-D-glucosamine, *Hep* L-*glycero*-D-*manno*-heptose, *P* phosphate

is acylated at two sugar residues located at the reducing end of the molecule. Thus, LPS is amphiphilic and, in physiological fluids, forms supramolecular aggregates that adopt inverted structures.

The structural characterization of LPS isolated from different gram-negative bacteria revealed that wild-type LPS of Enterobacteriaceae and Pseudomonadaceae are S-type LPS which, on the basis of structural features, genetic organization, biosynthesis, biological functions and biological activi-

ties, can usually be divided into three domains: the O-specific chain, the core region and lipid A. In contrast to S-type LPS, R-type LPS lack an O-specific chain and can be isolated from mutant bacteria with defects in LPS biosynthesis; therefore, they are unable to assemble complete LPS. Apart from these mutants, only R-type LPS have been identified in certain other non-enteric pathogenic bacteria, such as *Neisseria*, *Bacteroides*, *Haemophilus* and *Chlamydia* (RIETSCHEL et al. 1990). For most of these bacteria, it is unclear why they do not express an O-specific chain, which appears to be protective for other gram-negative bacteria. In *Neisseria*, the expression of neuraminic acid (Neu5NAc) as the terminal sugar in their LPS apparently helps this microorganism to evade an antimicrobial immune response. Mimicry of host antigens, in combination with other mechanisms (such as phase variation), may render the O-specific chain dispensable (BEYNON et al. 1992; JONES et al. 1992; PAVLIAK et al. 1993; YAMASAKI et al. 1993).

The chemical structure of LPS is subject to phase variation in different human pathogens, such as *N. gonorrhoeae* (VAN PUTTEN 1993), *N. meningitidis* (DE VRIES et al. 1996), *H. influenzae* (WEISER et al. 1989), *Helicobacter pylori* (APPELMELK et al. 1998), *Francisella tularensis* (COWLEY et al. 1996), *Coxiella burnetti* (VISHWANATH and HACKSTADT 1988) and *Legionella pneumophila* (LÜNEBERG et al. 1998). Whereas in *Neisseria* and *H. influenzae* (which produce an R-type LPS) the structure of the core-oligosaccharide is affected, it is the structure of the *O*-polysaccharide that is affected in *H. pylori*. Phase variation can be regarded as an adaptive mechanism of the pathogen to promote infection and allow survival in different environments. As an example, *N. gonorrhoeae* upregulates the amount of Neu5NAc on its surface LPS to evade host defense mechanisms by mimicking host antigens. At early stages of infection, the expression of sialic acid is downregulated, because a high surface density of Neu5NAc hinders the entry of *N. gonorrhoeae* into human mucosa cells (VAN PUTTEN 1993). Strains of *N. meningitidis* and *H. influenzae* also express similarly sialylated LPS. In addition, *H. influenzae* incorporates phosphorylcholine into its LPS (WEISER et al. 1998), a chemical structure often present on surfaces of infectious pathogens of the respiratory tract, such as *Mycoplasma fermentans* (ZÄHRINGER et al. 1997) and *Streptococcus pneumoniae*, or pathogens like *N. gonorrhoeae* and *N. meningitidis* (FARO et al. 1985; KOLBERG et al. 1997), which colonize other mucosal surfaces. The display of this structure enhances persistence on mucosal surfaces but renders *H. influenzae* more susceptible to the bactericidal activity of human serum. Mimicry of host antigens is employed by *H. pylori*, probably to evade host defense mechanisms by phase variation of the *O*-polysaccharide, which contains Lewis-X and Lewis-Y epitopes (MORAN and ASPINALL 1998). Infection with *H. pylori* and the display of these structures has been suggested to be connected to autoimmune disease by eliciting antibodies cross-reactive with self-antigens. Cross-reactive antibodies also seem to play a role in the development of the neurological disorder GBS, because core-oligosaccharides of certain *Campylobacter jejuni* serotypes associated with the disease contain structural motifs

also found in gangliosides (PRENDERGAST et al. 1998). However, a recent study (KOGA et al. 1998) investigating 35 patients serologically did not find a correlation between autoreactive antibodies and the development of GBS.

Shingomonas paucimobilis (KAWAHARA et al. 1991) contains glycosphingolipids that appear to replace LPS and, thus, together with *Borrelia burgdorferi* (TAKAYAMA et al. 1987), may constitute a new group of gram-negative microorganisms that do not require LPS for growth and multiplication (ZÄHRINGER et al. 1994).

I. Structural Characteristics of the O-Specific Chain

Bacteria with a smooth colony appearance attach a polysaccharide chain to the core region which, in many cases, is a heteropolymer made of repeating units (Fig. 3). These are smaller oligosaccharides two to eight monomeric sugar residues in size (JANN and JANN 1984; KNIREL and KOCHETKOV 1994a). As another example, a homopolymer of up to 75 molecules of legionaminic acid (5-acetamidino-7-acetamido-8-*O*-acetyl-3,5,7,9-tetradeoxy-D-*glycero*-L-*galacto*-non-2-ulosonic acid) has recently been described for *L. pneumophila* serotype 1 (KNIREL et al. 1994b). In enterobacterial LPS, these polysaccharide chains may contain up to 50 units (JANN and JANN 1984). The *O*-chain region of LPS may penetrate capsular and slime layers, which are frequently found on bacterial cells, rendering them accessible to binding proteins, such as serum factors and antibodies. Due to an overwhelming number of different sugars involved in the biosynthesis of O-specific chains (which are subject to further modification by, e.g., acetylation, methylation, phosphorylation, amidation and substitution with amino acids), bacterial cells produce a tremendously high number of different chemical structures, which are targets of specific antibodies (GALANOS et al. 1977). The recognized epitopes represent the *O*-antigenic determinants, also termed "factors". Therefore, LPS (as *O*-antigens) are of great diagnostic value and represent the structural basis of the "Kaufmann-White scheme" for serotyping *Salmonella* (KAUFFMANN 1978).

II. Structural Characteristics of the LPS Core

Chemical analysis of LPS obtained from *S. enterica* revealed that the same subset of carbohydrates was present in similar amounts in different serovars, whereas other carbohydrates were present in variable amounts. This led to the assumption that *Salmonella* LPS possess a common structure that carries the O-specific chain (GALANOS et al. 1977). This LPS domain, which connects the lipid-A domain to the O-specific chain, has been termed the core region. Chemical analysis of R-mutant *Salmonella* bacteria defective in LPS-biosynthetic pathways led to the differentiation of several chemotypes (R-chemotypes) that could be distinguished serologically (R-factors; GALANOS et al. 1977). In Enterobacteriaceae such, as *E. coli* and *S. enterica*, these chemotypes are referred to as Ra–Re. Ra describes the largest core

structure, and Re was assigned to the smallest core structure (Fig. 3), which is devoid of all core sugars except for a 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) disaccharide.

The core region of LPS consists of a complex oligosaccharide and, compared with O-specific chains, shows limited structural variability (HOLST and BRADE 1992). In the genus *Salmonella*, only two core types have been found (HOLST and BRADE 1992; OLSTHOORN et al. 1998) and, in *E. coli*, five different core types can be distinguished (HOLST and BRADE 1992). A typical structure present in LPS core structures of Enterobacteriaceae is a tetrasaccharide, α-D-glucose-(1→3)–L-α-D-heptose-(1→3)–L-α-D-heptose-(1→5)–Kdo, which is also found in LPS of *Yersinia*, *Salmonella*, *E. coli*, *Shigella* and *Citrobacter* (HOLST and BRADE 1992).

In enterobacteria and some other families, one can differentiate between an inner-core region and an outer-core region. The outer core contains predominantly pyranosidic hexoses, such as D-glucose, D-galactose, 2-amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-galactose (Fig. 3). Characteristic carbohydrate components of the inner core region are octulosonic acids and heptopyranoses (Hepp). These heptoses mainly possess the L-*glycero*-D-*manno* configuration (HOLST and BRADE 1992). The vast majority of analyzed LPS structures contain octulosonic acids in the form of Kdo in their inner-core regions (HOLST and BRADE 1992). Kdo has been shown to be replaced in some strains of *Acinetobacter* (KAWAHARA et al. 1987) and *Burkholderia cepacia* (KAWAHARA et al. 1994) by 2-keto-D-*glycero*-D-*talo*-octonic acid (Ko).

In all known LPS structures, the polysaccharide chain (core region and O-specific chain) is attached covalently to the lipid A component via either Kdo or Ko. Kdo is indispensable for the viability of LPS-containing gram-negative bacteria. The smallest LPS structure sufficient for bacterial growth and multiplication was isolated from a deep, rough mutant of *H. influenzae* (HELANDER et al. 1988). In this LPS, only one Kdo residue, phosphorylated in position 4 or 5, is attached to the lipid A. A negatively charged substituent of a different nature (such as hexuronic acid, Kdo or phosphate) at position 4 of the Kdo residue next to lipid A is always found and, thus, appears to be indispensable for bacterial viability. Therefore, a minimal structure of the core oligosaccharide with at least two negative charges seems to be important for the integrity of the outer membranes of gram-negative bacteria. Core structures with one (*H. influenzae*, *Bordetella pertussis*, *Vibrio cholerae*) or two Kdo residues (enterobacteria, pseudomonads) have been identified (HOLST and BRADE 1992). In addition, representatives of the genus *Chlamydia* have a unique, rough LPS consisting of a Kdo trisaccharide with the structure α-Kdo-(2→8)–α-Kdo-(2→4)–α-Kdo (BRADE et al. 1997). In addition, other saccharides of the inner-core region and non-stoichiometric substituents (such as phosphate and 2-aminoethanol phosphate and diphosphate) play an important role in the expression of the complete physiological capabilities of these microorganisms (SCHNAITMAN and KLENA 1993; WHITFIELD and VALVANO 1993).

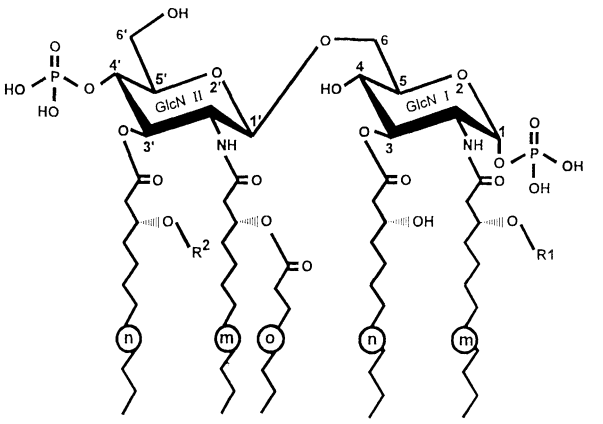
III. Structural Characteristics of Lipid A

The lipid anchor of the LPS molecule is called lipid A (ZÄHRINGER et al. 1994). In different bacterial groups, its architecture is (to some degree) structurally conserved, probably reflecting its essential role for the functioning of the outer membrane (RIETSCHEL et al. 1991). The linkage between the polysaccharide part and the lipid A is formed by Kdo (Fig. 3), which results in an extreme acid lability of this linkage. Therefore, lipid A was obtained in free form by weak acid hydrolysis of LPS (WESTPHAL and LÜDERITZ 1954) and became accessible to a detailed structural analysis (ZÄHRINGER et al. 1994).

Despite the presence of a common structural principle (represented by substituting fatty acids for the hydrophilic carbohydrate backbone of amino sugars), lipid-A preparations extracted from bacteria show some heterogeneity, i.e., mature and immature molecular species are present in the same culture. Identification of natural heterogeneity with respect to the lipid-A structure is of considerable biomedical and pharmaceutical interest due to the fact that lipid A is responsible for many of the toxic effects exerted by LPS, even at low concentrations (see below).

An exhaustive and detailed review of chemical structures of lipid A has been published recently (ZÄHRINGER et al. 1994). A lipid-A structure that is widely distributed in nature is present in *E. coli* LPS (Fig. 4). It consists of a 1,4′-bisphosphorylated, β-1,6-linked glucosamine (GlcpN) disaccharide (GlcpN-I–GlcpN-II lipid-A backbone) that is substituted by *R*-3-hydroxymyristic acid [14:0(3-OH)] residues in positions 2, 3, 2′ and 3′. Secondary acyl groups substitute the hydroxyl group of 14:0(3-OH) in positions 2′ (tetradecanoic acid, 14:0) and 3′ (dodecanoic acid, 12:0), leading to an asymmetric distribution of fatty acids. This type of lipid A is also the main species found in *Salmonella* and other enterobacterial and non-enterobacterial genera, such as *Haemophilus* and *Providencia* (ZÄHRINGER et al. 1994). In *Salmonella*, a hepta-acylated lipid-A species that carries hexadecanoic acid (16:0) as a secondary fatty acid at position 2 of GlcpN-I is also present in non-stoichiometric amounts. Secondary fatty acids have only rarely been found to substitute the 3-hydroxyl group of the acyl residue at position 3 of GlcpN-I (QURESHI et al. 1997). The attachment site of the core region is the hydroxyl group at position 6 of GlcpN-II (HOLST and BRADE 1992). Therefore, in *E. coli*, only one hydroxyl group of the carbohydrate-backbone disaccharide is free; it is located at position 4 of GlcpN-I. It is not known whether it is biologically significant that this hydroxyl group is not substituted.

Different types of lipid A are found in other non-enterobacterial LPS. Structural variability results from differences in the hydrophilic head group and in the hydrophobic acylation pattern (ZÄHRINGER et al. 1994). GlcpN residues constituting the hydrophilic carbohydrate backbone of *E. coli* lipid A may be replaced by 2,3-diamino-2,3-dideoxy-glucopyranose (GlcpN3N). The disaccharide has been found to be functionally replaced by a GlcpN3N monosaccharide in *Rhodopseudomonas palustris*, *R. viridis*, *Pseudomonas diminuta*



	Nature of		Number of carbon atoms		
	R^1	R^2	m	n	o
Escherichia coli	H	14:0	14	14	12
Haemophilus influenzae	H	14:0	14	14	14
Neisseria meningitidis	12:0	H	14	12	12
Chromobacterium violaceum	12:0	H	12	10	12

12:0, dodecanoic acid 14:0, tetradecanoic acid

Fig. 4. Primary structure of lipid-A components of different gram-negative bacteria expressing endotoxically active lipopolysaccharides

and *Phenylobacterium immobilis* whereas, in *C. jejuni*, *Brucella*, and Chromatiaceae (KASAI et al. 1990; MAYER et al. 1990; MORAN et al. 1991), the β-1,6-linked disaccharide structure is retained and either only GlcpN-II or both GlcpN residues are replaced by GlcpN3N. GlcpN3N has never been found to replace GlcpN-I in a hybrid disaccharide.

Whereas the non-glycosidic phosphate may be absent, e.g., in *Bacteroides* (WEINTRAUB et al. 1989), the glycosidic phosphate has always been found to be present, with a single exception (BHAT et al. 1994). Furthermore, both phosphates may carry either of the phosphates, 2-aminoethanol, 2-aminoethanol phosphate, GlcpN, 4-amino-4-deoxy-L-arabinopyranose or D-arabinofuranose.

A peculiar situation has been encountered in *Rhizobium leguminosarum*, whose lipid A lacks phosphate (BHAT et al. 1994). Instead, GlcpN-I is converted into 2-aminogluconate, and the 4'-phosphate is replaced by a galacturonic acid residue after enzymatic cleavage of this phosphate by a specific phosphatase (PRICE et al. 1995). Thereby, the number of negative charges is retained, and phosphate groups are functionally replaced by carboxyl groups.

Lipid A containing a disaccharide as the hydrophilic backbone may carry four to seven fatty acids which, if present in an even number, may be either symmetrically or asymmetrically distributed over the disaccharide backbone. The acyl groups found in LPS may be saturated or may carry double bonds or hydroxy or keto groups (MAYER et al. 1990; ZÄHRINGER et al. 1994). A characteristic feature of LPS is the presence of fatty acids hydroxylated at C-3 (the β-position). However, 2-(or α-)hydroxylated fatty acids are also found frequently. The 3-(or β-)hydroxylated fatty acids identified so far include fatty acids of the *R* configuration and possess a chain ranging from ten (as found in *P. aeruginosa*) to 22 carbon atoms (as present in *Chlamydia*; ZÄHRINGER et al. 1994) in length. They were directly attached to the hydrophilic backbone by ester or amide linkages and are referred to as primary fatty acids. 3-Hydroxylated fatty acids were frequently esterified by secondary fatty acids, as described above for *E. coli*-type lipid A. In contrast, 2-hydroxylated fatty acids occur less frequently and always possess the *S*-configuration (RIETSCHEL 1976). So far, they have not been found in primary positions and have not been found to be further esterified. The degree of 2-hydroxylation appeared to be regulated by environmental factors, such as growth temperature, and structural analysis suggested that α-hydroxylation takes place at the fully acylated lipid A (ZÄHRINGER et al. 1994). In rare cases, amide-linked fatty acids may be 3-keto-fatty acids, as observed in LPS of bacteria that phylogenetically belong to the α-3 subgroup, such as *Rhodobacter capsulatus*, *R. sphaeroides*, *Paracoccus denitrificans* and *V. anguillarum* (MAYER et al. 1990). Unsaturated fatty acids, which are rarely found in LPS, are also present in lipid A from *R. sphaeroides*, *R. capsulatus* and *P. denitrificans* and in lipid A of *E. coli* grown at low temperature.

The distribution of secondary fatty acids (which, in *E. coli*, are attached after biosynthesis of the core region has been initiated; RAETZ 1996) determines the symmetry of enterobacterial lipid A. Attachment of secondary fatty acids in positions 2′ and 3′ leads to an asymmetrical lipid-A molecule. Examples of symmetrical lipid-A structures are found in *Chromobacterium violaceum*, *Rhodocyclus gelatinosus* and *N. meningitidis* (Fig. 4; ZÄHRINGER et al. 1994). *C. violaceum*, as a typical representative, possesses a hexa-acylated lipid A, which is composed of a 1,4′-bisphosphorylated, β-1,6-linked GlcpN disaccharide, as found in *E. coli*, and which has primary-fatty-acid substitutions of 12:0(3-OH) at positions 2 and 2′ and 10:0(3-OH) at 3 and 3′. Secondary 12:0 are present in positions 2 and 2′. The 12:0 in position 2′ is α-hydroxylated in non-stoichiometric amounts, leading to microheterogeneity.

C. Biosynthesis of LPS

The genes involved in the biosynthesis of LPS (Mäkelä and Stocker 1984; Schnaitman and Klena 1993; Raetz 1996) have been localized and identified in *Salmonella* and *E. coli*. The genes encoding the enzymes involved in the biosynthesis of the core region and the O-specific chain are grouped in clusters previously termed *rfa* and *rfb*, respectively. Due to the increasing number of genes that have been identified, a new nomenclature for the genes involved in the biosynthesis of bacterial polysaccharides has been proposed (Reeves et al. 1996). According to this scheme, the *rfa* locus is now referred to as *waa*, and genes involved in *O*-antigen biosynthesis belong to a group of genes termed *wba–wby*.

I. Biosynthesis of Lipid A

Biosynthesis of lipid A (Raetz 1996) proceeds via common intermediates, which have been identified in *E. coli* (Nishijima and Raetz 1981; Takayama et al. 1983) and *Salmonella* (Hansen-Hagge et al. 1985) and have been termed lipid X and precursor Ia (in *E. coli*) and lipid Y and precursor Ib (in *Salmonella*; Fig. 5). Lipid X and lipid Y are both formed from undecaprenylpyrophosphorylyl (UdP)-activated phosphorylated *N*-acetyl-D-glucosamine; after *O*-acylation in position 3, de-*N*-acetylation and *N*-acylation in position 2, they are deactivated to form the respective acylated monosaccharide units. In *E. coli*, a specific disaccharide synthetase then causes one molecule of UdP-activated 2,3-diacyl-glucopyranosylamine to condense with one molecule of lipid X via a β-1,6-linkage, giving rise to precursor Ia. Lipid Y differs from lipid X in that it contains an additional 16:0 as a secondary fatty acid at position 2. In *Salmonella*, lipid Y has been found to partially replace lipid X in the condensation reaction. Biosynthesis of lipid-A structures as different as those found in *R. leguminosarum* and *E. coli* proceeds via the same precursor molecules (Price et al. 1995), suggesting that lipid-A biosynthesis follows a highly conserved pathway.

II. Biosynthesis of the Core Region

The biosynthesis of the core region is initiated before the biosynthesis of lipid A is completed. This fact may be the reason mutants that lack Kdo are not viable under normal growth conditions. The initial step is the transfer of one Kdo residue from cytosine monophosphate (CMP)-activated Kdo to position 6′ of precursor Ia (*E. coli*) or Ib (*Salmonella*). Kdo is formed from arabinose 5-phosphate and phosphoenolpyruvate, with subsequent removal of the phosphate group from the intermediate Kdo-8-phosphate. Remarkably, as shown for *H. influenzae* (White et al. 1997), *E. coli* (Belunis and Raetz 1992b) and *Chlamydia* (Belunis et al. 1992a; Mamat et al. 1993; White et al. 1997), the transfer of each Kdo residue is accomplished by a single (and, thus, multi-

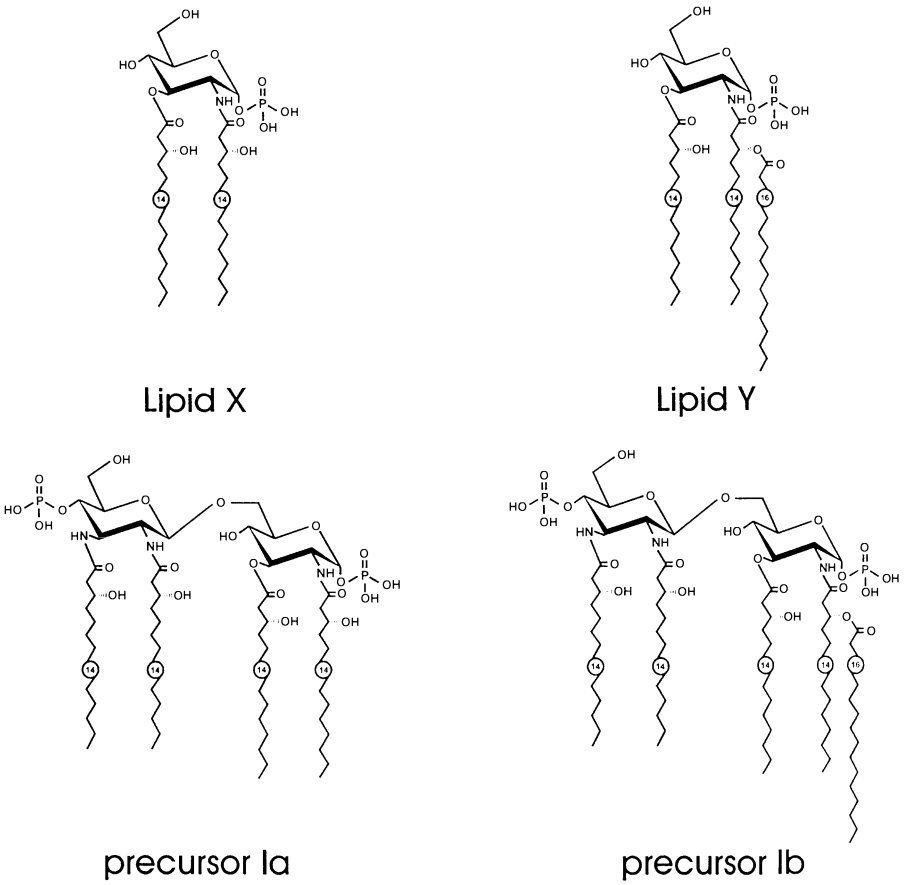


Fig. 5. Chemical structure of precursor molecules involved in biosynthesis of *Escherichia coli* (lipid X and precursor Ia) and *Salmonella* (lipid Y and precursor Ib) lipopolysaccharides

functional) Kdo transferase. Following the transfer of Kdo, secondary fatty acids are attached to the β-hydroxy groups of primary fatty acids of lipid A. The biosynthesis of the core region is then completed in a stepwise manner by transfer of activated monosaccharides, a process catalyzed by glycosyl transferases specific for a single acceptor structure.

III. Biosynthesis of the O-Specific Chain

Biosynthesis of the O-specific chain in enterobacteria like *E. coli*, *S. enterica*, *Shigella* and *Klebsiella* can proceed in two ways (Mayer et al. 1989). In *rfbP*-dependent biosynthesis, repeating units are assembled on UdP, and a completed unit is then transferred onto an UdP carrier molecule carrying one

complete repeating unit. Therefore, elongation proceeds at the reducing end by sequential transfer of the growing chain on newly synthesized repeating units. Alternatively, in *rfe*-dependent biosynthesis, chain-elongation is achieved stepwise, by sequential transfer of activated monosaccharides on UdP at the non-reducing end of the chain.

D. Structure–Activity Relationships of LPS and Lipid A

The preparation and structural elucidation of lipid A from different bacteria and the chemical synthesis of defined partial structures made it possible to determine the molecular parameters governing endotoxic activity. Among a variety of parameters, the release of pro-inflammatory cytokines from murine macrophage cell lines and human peripheral monocytes in response to lipid A and lipid-A partial structures was analyzed. Data from studies in the human system carried out in our laboratories (Loppnow et al. 1989a; Ulmer et al. 1992; Flad et al. 1993) are summarized in Fig. 6. It was demonstrated that the structure of the hydrophilic saccharide backbone (β-1,6-linked GlcpN or GlcpN3N disaccharide) is a fundamental structural principle required for the expression of biological activity (cleavage of the glycosidic bond caused the activity to decrease by a factor of 10^7) and that its phosphorylation had a decisive influence on endotoxicity (causing the activity to decrease by a factor of 10^2–10^4). The replacement of phosphate groups of the lipid-A backbone by a carboxymethyl group did not lead to a significant reduction of biological activity (Schromm et al. 1998). Thus, it appears that the presence of negative charges fulfils the requirements for biological activity. The loss of secondary fatty acids dramatically reduced the biological activity (by a factor of more than 10^7). Recently, these findings could be confirmed by using mutants of *E. coli* K-12 (Somerville et al. 1996) and *H. influenzae* (Nichols et al. 1997) in which the gene for an acyl transferase for secondary fatty acids had been inactivated. The strains are viable at 30°C, because the transfer of the Kdo residues and further biosynthesis of the core oligosaccharide only needs a lipid-A precursor molecule with four fatty acids. As expected, these mutants express an LPS with a significantly reduced biological activity. In addition, lipid-A preparations with an asymmetrical distribution of the secondary fatty acids show an increased endotoxicity in comparison to corresponding synthetic analogs that have symmetrical acylation (where the fatty acids in both cases have the same chain length).

Lipid A and LPS are amphiphilic molecules forming supramolecular structures in an aqueous medium above their critical micellar (aggregate) concentrations (CMC or CAC). However, the aggregate structure is determined by both the primary chemical structures of the molecules and environmental parameters, such as temperature, water content, pH value and the presence of divalent cations. By determination of the aggregate structures of defined lipid-A preparations under almost physiological conditions (37°C, pH = 7, >90%

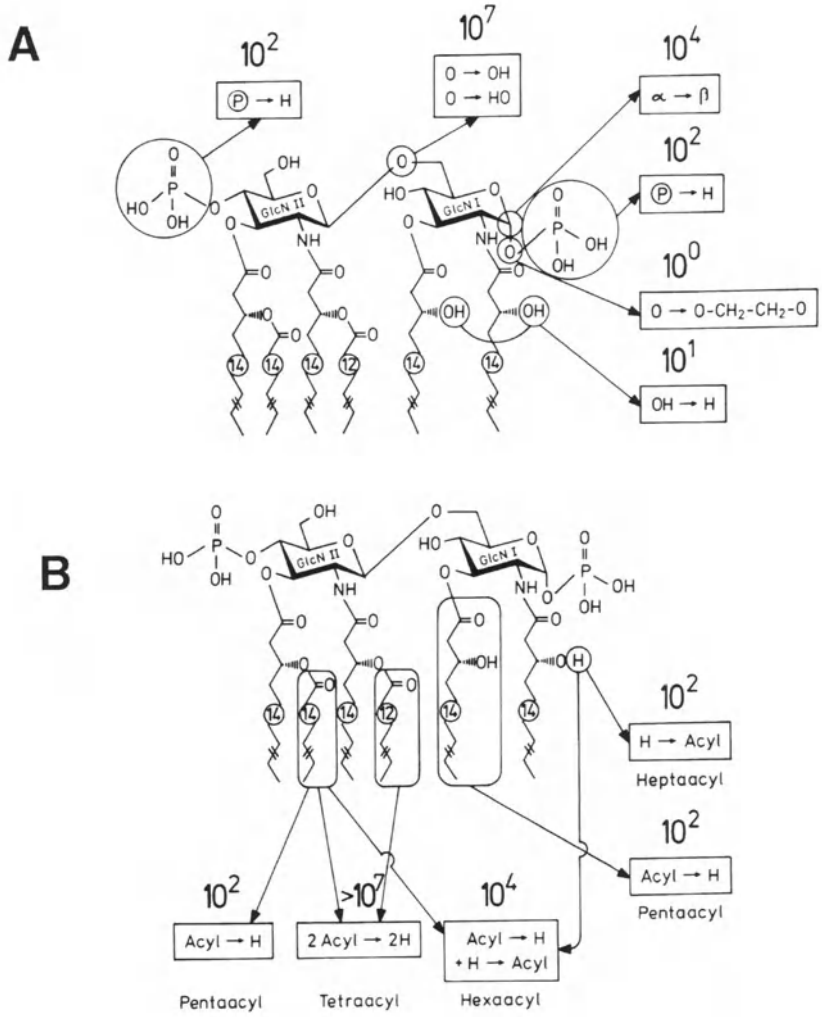


Fig. 6. Schematic representation of the relationship between the chemical structure and endotoxic activity of lipid A. Shown are chemical alterations of lipid A from *Escherichia coli* and the relative decrease of biological activity associated with them. **A** Modifications of the hydrophilic region (phosphorylated glucosamine disaccharide) of lipid A. **B** Modifications of the hydrophobic region (fatty acids) of lipid A

water content, presence of Mg^{2+}; Brandenburg et al. 1993, 1996; Seydel et al. 1993), it could be demonstrated that endotoxic active molecules have a distinct tendency to form cubic (Q) or inverted-hexagonal (H_{II}) supramolecular structures (Fig. 7). By determining the type of aggregate structure preferentially formed, the geometrical shape (conformation) of the lipid-A molecules can be deduced. Thus, in lipid-A molecules forming inverted structures, the

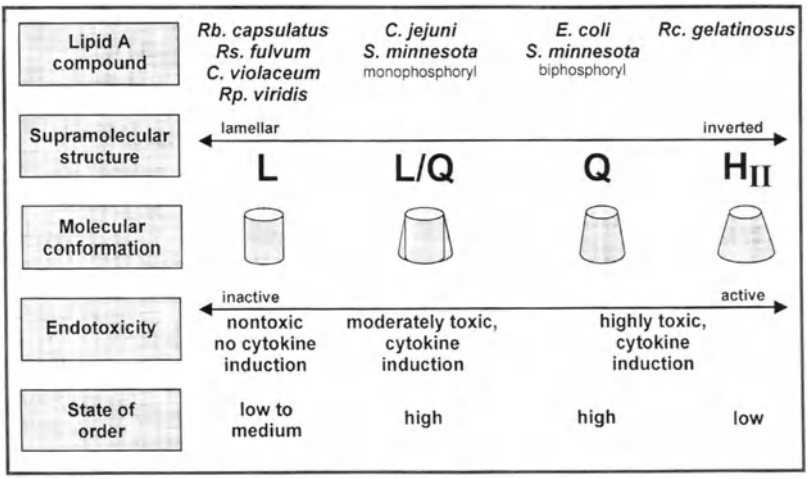


Fig. 7. Physical structure of endotoxically active and inactive lipid A. Endotoxically inactive lipid A possesses a cylindrical conformation and forms lamellar (L) aggregate structures under approximately physiological conditions. In contrast, biologically active lipid-A molecules show a truncated conical shape, which is enlarged in the hydrophobic region of the fatty acids and, therefore, leads to the formation of cubic (Q) or inverted hexagonal (H_{II}) aggregate structures

hydrophobic fatty-acid portion possesses a larger volume and adopts a conical shape. In contrast, biologically inactive lipid-A molecules, e.g., from *R. capsulatus*, are cylindrically shaped and associate with lamellar (L) aggregate structures. The molecular shape of biologically active lipid A corresponds to a truncated, cone-like conformation, which we call the "endotoxic conformation" (Rietschel et al. 1987) and which represents the active counterpart of humoral and cell-bound receptor molecules of the host organism. It had been assumed that the biological effect of LPS in physiological solutions was mediated by larger aggregates (Shnyra et al. 1993). Newer findings (Takayama et al. 1994; Schromm et al. 1995; Schromm et al. 1996; Yu and Wright 1996), however, show that rather small oligomeric aggregates or monomers take part in cell activation by endotoxins (Brandenburg et al. 1993). Therefore, the formation of a certain kind of supramolecular aggregate represents an intrinsic property of biologically active LPS molecules but is not necessarily a prerequisite for the expression of biological activity.

E. Cellular and Humoral Responses to LPS in Mammals

LPS-dependent activation was described for many cell types from different mammalian organisms. Cells of the immune system (in particular monocytes, macrophages, lymphocytes and endothelial cells) represent targets of prime importance. Each cell type reacts to endotoxin in a specific way, e.g., cell dif-

ferentiation and/or cell proliferation, increased phagocytosis or production of mediator or effector molecules.

As the first line of defense at the beginning of an infection, circulating *monocytes* and tissue-bound *macrophages* (Fig. 2) contact bacteria or bacterial products, such as endotoxins, and initiate the formation of an inflammatory reaction. In the presence of very low concentrations of LPS (<1 ng/ml serum), macrophages and monocytes produce a great number of different mediators, such as IL-1, IL-6, IL-8, IL-10, IL-12, IL-18, the migration-inhibitory factor (BERNHAGEN et al. 1993) and, most importantly, TNF-α (NATHAN 1987). These mediators contribute to the accumulation and activation of further effector cells of the immune system at the site of infection (BERNHAGEN et al. 1993; MASTROENI et al. 1993). In addition, the general defense mechanism of the host organism is activated by moderate fever, and the production of acute phase proteins by liver cells contributes to the antimicrobial defense. In addition to protein-like cytokines, monocytes and macrophages produce immunomodulatory lipids, such as metabolites of the arachidonic-acid pathway (leukotrienes, prostaglandins; LÜDERITZ et al. 1989; SCHADE et al. 1989; VOGEL 1990; BERNHAGEN et al. 1993), derivatives of linoleic acid (SCHADE et al. 1987) and platelet-activating factor (PAF; BRAQUET et al. 1987). Furthermore, endotoxins stimulate the phagocytic potency of macrophages and the production of reduced oxygen molecules (superoxide anion, hydrogen peroxide, hydroxyl radicals, nitric oxide; Fig. 2), thereby contributing to the killing of bacteria and the triggering of local or generalized inflammatory reactions. Thus, these mediators, released in small amounts, cause effective limitation of the infection and elimination of the health-threatening microorganisms (ECHTERNACHER et al. 1990; MASTROENI et al. 1993). However, overproduction of pro-inflammatory cytokines and reactive intermediates results in damage to host cells and organs (PARILLO 1993). Thus, the same cells that are important for the antimicrobial defense may play a key role in the development of sepsis.

Granulocytes (polymorphonuclear leukocytes) take up bacteria and cell-wall fragments, and their phagocytic activity is dramatically increased by endotoxins (SCHADE et al. 1987). Activated granulocytes augment the local inflammatory response in the early phase of an infection by sticking to the endothelial lining of blood capillaries and emigrating into the surrounding tissue by diapedesis. Thus, in the course of sepsis, they may cause damage to vessels and tissues. Granulocytes also contain enzymes (acyloxyacyl hydrolases and phosphatases) that degrade LPS into biologically inactive partial structures by specifically removing secondary fatty acids and phosphate groups (MUNFORD and HALL 1989). Activated by endotoxins and/or pro-inflammatory cytokines, granulocytes release polycationic proteins from endosomal compartments; these proteins can specifically bind to LPS. Among these, the bactericidal permeability-increasing protein (BPI) is the most completely characterized. It constitutes a 55-kDa membrane-associated protein present in the primary granules of neutrophilic granulocytes. It displays cytotoxic activity towards gram-negative bacteria and has an endotoxin-neutralizing capac-

ity (Mannion et al. 1989; Marra et al. 1990, 1992). Analysis of recombinant fragments and subsequent structural analysis of BPI allowed the identification of a lipid-A/LPS-binding site in the amino-terminal domain (Gray et al. 1989; Meszaros et al. 1993). A recombinant 23-kDa fragment ($rBPI_{23}$) effectively inhibits the LPS-dependent release of TNF-α, IL-1, IL-6 and IL-8 from human blood cells (Weiss et al. 1992; Meszaros et al. 1993) and protects animal and human volunteers from phlogistic endotoxemia.

In addition to cells of the immune system, *vascular cells* (endothelial and smooth-muscle cells) can also be stimulated by LPS. In these cells, stimulation yields the production of different cytokines, such as IL-1, IL-6, IL-8 and TNF-α (Loppnow and Libby 1989b, 1990a; Schönbeck et al. 1994, 1995), and of prostaglandins, nitric oxide, PAF and growth factors for blood cells (Libby et al. 1991; Mantovani and Bussolino 1991). In addition to these mediators, endothelial cells express adhesion molecules after exposure to LPS (Yu et al. 1986; Doherty et al. 1989); these cells, e.g., facilitate the attachment of blood platelets, granulocytes and monocytes to the vessel wall and support their emigration to the subjacent tissue.

The 53-kDa glycoprotein CD14 was identified as a LPS-binding molecule on the surface of monocytes, macrophages, and (in low concentrations) neutrophilic granulocytes (Wright et al. 1990). It is found on all mature myeloid cells (Griffin et al. 1981; Haziot et al. 1988; Wright et al. 1990; Ziegler-Heitbrock and Ulevitch 1993). The protein has no membrane-spanning region but is integrated into the cell membrane via a glycosyl-phosphatidylinositol (GPI) anchor. In addition to membrane-bound CD14 (mCD14), soluble isoforms of the protein (sCD14) weighing 55 kDa, 53 kDa or 48 kDa are found in serum (2–6 mg/ml in the circulation of healthy humans; Bazil et al. 1989; Frey et al. 1992); in CD14-positive cells, these isoforms are created by post-translational processing or enzymatic cleavage of cell-surface CD14 (Bazil and Strominger 1991; Derieux et al. 1994). sCD14 plays an important role in endotoxin activity in that it mediates the endotoxin-induced activation of CD14-negative endothelial cells (Frey et al. 1992; Haziot et al. 1993a; Pugin et al. 1993; Arditi et al. 1994; Goldblum et al. 1994). The special role of mCD14 in LPS-mediated cell activation (Fig. 8) has been demonstrated in different experimental systems (Wright et al. 1990; Lee et al. 1992; Ziegler-Heitbrock and Ulevitch 1993; Haziot et al. 1993b, 1996). The endotoxin-dependent release of TNF-α from monocytes, for example, can be inhibited by certain monoclonal antibodies against CD14 (Wright et al. 1990). After transfection to the CD14-gene into B-cell line 70Z/3, a direct association between sensitivity to endotoxin and the expression of CD14 can be demonstrated (Lee et al. 1992). Furthermore, CD14-deficient mice survive log-fold-higher endotoxin doses than the initial isogenic strain (Haziot et al. 1996). LPS binds to a high-affinity, amino-terminal domain of the CD14 molecule via the lipid-A portion (Juan et al. 1995; Viriyakosol and Kirkland 1995). Interestingly, cell-wall components of gram-positive bacteria (such as peptidoglycan, arabinogalactan and lipoarabinomannan) also stimulate CD14-dependent

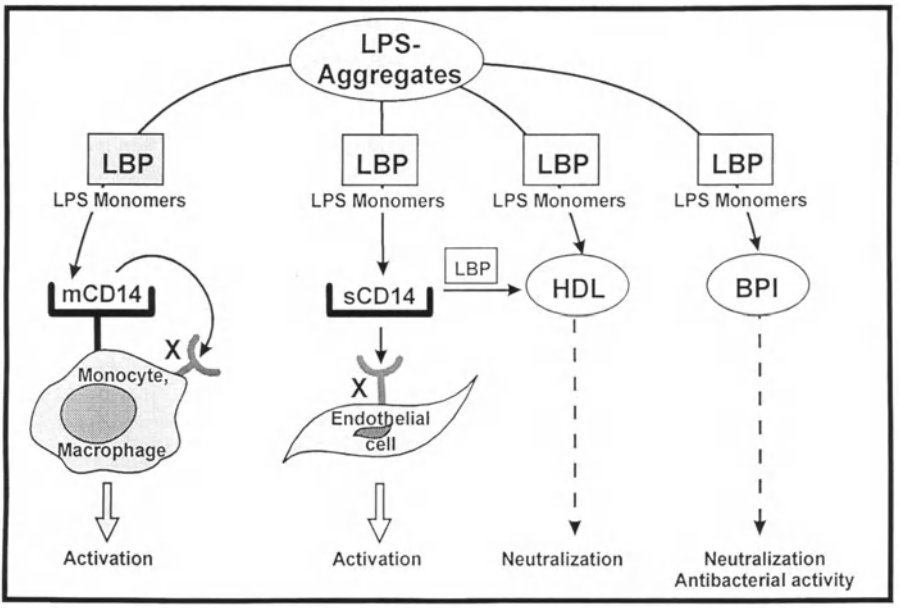


Fig. 8. Early stages of cell activation by endotoxins. The effects of lipopolysaccharides (*LPS*) are modulated by primary binding to serum proteins. In this process, LPS-binding protein (*LBP*) has an important function by liberating smaller oligomers and/or monomers from LPS aggregates and transmitting them to receptor molecules. Several mechanisms for the activation of target cells, where CD14 is of decisive importance in cases of low endotoxin concentration (<10 ng/ml), are discussed. A transmembrane, signal-transmitting receptor for LPS (here termed X) is not yet known. *BPI* bactericidal, permeability-increasing protein, *HDL* high-density lipoprotein, *mCD14* membrane-bound CD14 molecule, *sCD14* soluble (serum) CD14 molecule

activation of monocytes (Weidemann et al. 1994, 1997). Based on these findings, it was proposed that CD14 represents a pattern-recognition receptor (Pugin et al. 1994). However, the structural prerequisites for cell binding of these molecules and the precise role of CD14 in these systems are not known in detail. Nevertheless, mutational analysis of mCD14 transfected into 70Z/3 cells has implicated subtle differences in the activation of these cells by peptidoglycan and LPS; these differences were suggested to result because cells recognize these two substances differently (Kirkland and Viriyakosol 1998).

The activation of cells at low concentrations of endotoxin is augmented approximately 1000-fold in the presence of serum (Wright et al. 1990; Haziot et al. 1993a; Goldblum et al. 1994). This effect is due to the action of LPS-binding protein (LBP; Schumann et al. 1990; Schumann 1992). LBP is a 58-kD glycoprotein synthesized in hepatocytes and constantly released into the bloodstream (Tobias and Ulevitch 1993). Its serum concentration of approximately 10–20 μg/ml can rise to 200 μg/ml after infection, during the host's acute-phase response. LBP shows 44% sequence similarity to BPI and, like

BPI, has a high-affinity, amino-terminal binding region for LPS and lipid A (Schumann et al. 1990; Tobias and Ulevitch 1993). The catalytic mechanism underlying the action of LBP has been examined intensively (Hailman et al. 1994; Tobias et al. 1995; Schromm et al. 1996; Yu and Wright 1996). It is currently believed that LBP binds to LPS aggregates and changes their supramolecular structures into LPS oligomers or monomers (Fig. 8). Complexed with LBP, LPS then binds to cell-bound and/or soluble CD14. Additionally, LBP catalyzes the transfer of LPS to BPI and to high-density lipoprotein (Flegel et al. 1993; Wurfel et al. 1994; Horwitz et al. 1995; Yu and Wright 1996), indicating that LBP also participates in the neutralization of endotoxins. Thus, it is conceivable that, similar to the case in sCD14, low amounts of LBP augment LPS activity, whereas high doses inhibit LPS activity (Lamping et al. 1998).

The fact that LBP is continuously produced at high levels during infection has led to the assumption that LPS–LBP complexes are consumed by phagocytic cells (Gegner et al. 1995). Indeed, such an uptake, which depends on the presence of serum and CD14, has been demonstrated (Pugin et al. 1993). Thus, LBP may also be involved in the clearance of LPS (and bacteria) from the circulation. The use of monoclonal anti-LBP antibodies provided evidence that cellular activation and LBP-promoted LPS uptake are independent events (Tapping et al. 1998) and that only a small proportion of mCD14 is actually involved in cell activation, whereas the majority of mCD14 is involved in LBP–LPS binding, eventually leading to uptake. Blood from LBP-gene-deficient ($LBP^{-/-}$) mice was shown to be hyporesponsive to LPS ex vivo (Wurfel et al. 1997). However, systemic administration of LPS led to similar responses in $LBP^{-/-}$ and heterozygous $LBP^{+/-}$ mice (Fenton and Golenbock 1998). It was concluded that $LBP^{-/-}$ mice respond to systemic LPS in a CD14-dependent but LBP-independent manner. It was suggested that either high local concentrations of sCD14 in tissues or another transfer molecule (rather than LBP/mCD14) may be involved in activation (Fenton and Golenbock 1998). $LBP^{-/-}$ mice were found to clear LPS from the circulation rapidly and to be resistant to LPS-induced death if pre-treated with galactosamine (Jack et al. 1997). That a different situation is encountered in systemic administration of endotoxin compared with infections with live bacteria is evident from the observation that LBP-expressing mice show increased survival compared with LBP knockout mice after challenge with live bacteria (Jack et al. 1997).

Although the importance of CD14 for specific binding to LPS and cell activation is indisputable, the signal-transduction mechanisms following binding are less clear. Because the CD14 protein possesses no domain spanning the cytoplasmic membrane (Haziot et al. 1988) and, in its soluble form, even lacks the GPI anchor (Frey et al. 1992; Haziot et al. 1993a; Pugin et al. 1993; Goldblum et al. 1994; Arditi et al. 1994), the presence of an additional signal-transducing transmembrane protein has been postulated. It is known that high endotoxin concentrations (>100 ng/ml) can also stimulate macrophages and monocytes to release cytokines in a CD14-independent

manner (Lynn et al. 1993; Proctor et al. 1994; Ingalls and Golenbock 1995). This finding was confirmed using macrophages from a CD14-negative mouse strain (Perera et al. 1997). The involvement of purinoreceptors in endotoxic action derives from the observation that adenine nucleotides protect mice from lethal challenges of endotoxin and inhibit the release of toxic mediators, such as TNF-α and IL-1 (Proctor et al. 1994). In addition, pre-treatment of macrophages with 2-methylthioadenosine trisphosphate reduced the amount of NO generated after subsequent LPS treatment (Hu et al. 1998). However, other endotoxic activities, such as pro-coagulant activity, are unaffected and indicate a modulation of specific pathways by adenine nucleotides. Recently, CD11c/CD18, a heterodimeric adhesion molecule of the integrin family, has been described as a functional transmembrane receptor mediating CD14-independent stimulation of macrophage-like cells in the presence of high LPS concentrations (Ingalls and Golenbock 1995). However, deletion of its membrane-spanning domain did not inhibit its function (Ingalls et al. 1997). It should be emphasized that LPS concentrations in these experiments were distinctly higher than those observed during septic episodes.

In the search for a membrane signal transducer for CD14-dependent cell activation, a ligand-binding test that permits the detection of the binding of lipid A to membrane proteins separated by gel electrophoresis and immobilized by transfer to nitrocellulose membranes has been developed (Schletter et al. 1995). With this method, an 80-kDa protein that binds lipid A only in the presence of serum or a mixture of purified sCD14 and LBP can be isolated. The protein was found in cell membranes of human peripheral blood monocytes, endothelial cells and the human monocyte cell line Mono MAC 6. It was recently identified as decay-accelerating factor (DAF; El-Samalouti et al. 1998). Whether DAF is involved in LPS-induced or LBP/CD14-dependent signal transduction remains to be elucidated. Very recently, the Toll-like receptor TLR-2 was shown to be involved in LPS-induced cell activation (Yang et al. 1998). The TLR-2 cytoplasmic domain is essential for activation, and TLR-2 function depends on LBP and is enhanced by CD14.

LPS binding results in the rapid activation of protein tyrosine kinases (PTKs). The PTKs can be divided into two groups based on their predicted structures. One group, which possesses extracellular domains that generally bind polypeptide hormones, are the "receptor PTKs", and the second major group, which lacks extracellular sequences, are considered "nonreceptor PTKs" (Bolen et al. 1992). There appear to be at least eight distinct families of nonreceptor PTKs; these include the Src family, which consists of at least nine members: Src, Yes, Fyn, Lyn, Lck, Hck, Fgr, Blk and Yrk. The kinases Hck, Fgr and Lyn are strong candidates for the primary signal transducers of LPS responses (Boulet et al. 1992; Stefanova et al. 1993). All three of these kinases are rapidly activated after LPS treatment, and a portion of intracellular Lyn directly associates with CD14 (Stefanova et al. 1993). However, despite the biochemical evidence suggesting that Hck, Fgr and Lyn are involved in LPS signal transduction, macrophages derived from $Hck^{-/-}$ $Fgr^{-/-}$ $Lyn^{-/-}$ mice have

normal LPS functional responses, indicating that, at least in mice, the kinases Hck, Fgr and Lyn are not obligatory for LPS-initiated signal transduction (MENG and LOWELL 1997). Although the PTK specifically involved in LPS signaling is unknown, one candidate is the C-Abl tyrosine kinases (LE et al. 1998). Studies utilizing a range of PTK inhibitors indicate that PTKs play a central role in LPS-induced production of TNF-α and IL-1β and in activation to a tumoricidal state (WEINSTEIN et al. 1992; DONG et al. 1993a; SHAPIRA et al. 1994). Among the most prominent tyrosine-phosphorylated proteins in LPS-stimulated macrophages are the p42 and p44 isoforms of mitogen-activated protein kinase (MAPK), also known as extracellular-signal-regulated kinase 1 (ERK1)/ERK2 (WEINSTEIN et al. 1992; DONG et al. 1993b). MAPKs, found in the cytoplasm and nuclei of cells, are a family of 40- to 45-kDa serine/threonine kinases that participate in signaling pathways initiated by many extracellular stimuli. MAPKs are activated by phosphorylation on both tyrosine and threonine residues (BLUMER and JOHNSON 1994). This reaction is catalyzed by MEK (MAPK/ERK kinase), whose activation also requires serine/threonine phosphorylation (BLUMER and JOHNSON 1994). In turn, MEK is activated by RAF-1, a serine/threonine-specific kinase reported to participate in the response to LPS (GEPPERT et al. 1994). RAF-1 may participate in the activation of MAPK via both ras-dependent and -independent mechanisms in macrophages (BUSCHER et al. 1995).

In addition to ERK1 and ERK2, p38 the mammalian homologue of the yeast MAPK-like kinase HOG1 (WEINSTEIN et al. 1992; HAN et al. 1994) and c-Jun kinase (HAMBLETON et al. 1996) become phosphorylated (and, thereby, enzymatically activated) within minutes after LPS treatment in macrophages (HAN et al. 1994). There are arguments that the MAPK pathway is not the only mechanism mediating cellular responses to LPS. By introducing a regulable form of RAF-1 that can activate ERK1/2 independently of LPS into murine macrophages, it was shown that activation of ERK1/2 by RAF-1 is not sufficient to induce all the biological responses triggered by LPS (HAMBLETON et al. 1995). According to other studies, further MAPK-related kinases, such as stress-activated protein kinase, c-Jun N-terminal kinase and p38, are reasonable candidates for MAPK-independent elements that mediate LPS signalling in macrophages (SANGHERA et al. 1996).

Because of the predominant role of serine/threonine kinases in LPS signalling, a critical role of serine/threonine phosphatases in the monocyte response to LPS has been postulated (BARBER et al. 1995). By the use of calyculin A, a potent inhibitor of protein phosphatases PP1 and PP2A (two of the most abundant serine/threonine phosphatases in the cell), it was shown that the LPS-signalling pathway has at least two serine/threonine phosphatase requirements, distinguishable by their time-dependent sensitivities to calyculin A (BARBER et al. 1995). Several studies suggest that treatment of macrophages with LPS results in the activation of phospholipase C, as determined by the formation of inositol trisphosphate ($InsP_3$). The response is modest and occurs 1–20 min after activation (PRPIC et al. 1987). This slow turnover of $InsP_3$ is not

accompanied by a rise in intracellular calcium levels (DRYSDALE et al. 1987; HURME et al. 1992). In our experiments, LPS induced small $[Ca^{2+}]_i$ increases that elicited only a weak dose dependency (HAUSCHILDT et al. 1990). In addition to $InsP_3$, diacylglycerol (DAG) is generated as a result of the activation of phospholipase C. DAG is a natural activator of protein kinase C (PKC), a serine/threonine kinase first characterized on the basis of its activation in vitro by Ca^{2+}, phospholipid and DAG (DEKKER and PARKER 1994). PKC is also the major receptor for phorbol esters, which activate the kinase in vitro in a manner similar to its activation by DAG (DEKKER and PARKER 1994). PKC is not a single molecular entity but consists of a large number of related proteins. These PKC isotypes show differences in cofactor dependence and responsiveness to phospholipid metabolites (DEKKER and PARKER 1994). Apart from the Ca^{2+}- and phospholipid-dependent protein kinase C, which has been shown to be involved in LPS signal transduction (SHAPIRA et al. 1994) and the induction of tumoricidal properties in macrophages (NOVOTNEY et al. 1991; DONG et al. 1993a), the DAG-independent and phorbol ester-insensitive PKC isoform ξ has also been implicated in LPS responsiveness (FUJIHARA et al. 1994). However, as there are numerous reports showing LPS-mediated responses without PKC activation (DONG et al. 1989; GLASER et al. 1990), the role of PKC in LPS-induced signal transduction remains controversial.

Some evidence indicates that the interaction of LPS with cells results in activation of pertussis toxin (PT)-sensitive, guanine-nucleotide-binding (G) proteins (JAKWAY and DEFRANCO 1986; DANIEL-ISSAKANI et al. 1989). PT, which adenosine-diphosphate-ribosylates the α-subunits of Gi proteins, thereby inhibiting their action, has been used as a tool to study G-protein-mediated signal transduction. PT has been shown to inhibit LPS-induced IL-1 production of the pro-monocytic cell line U937 and the macrophage cell line P388 D_1 (JAKWAY and DEFRANCO 1986; DANIEL-ISSAKANI et al. 1989). It was found to enhance LPS-induced TNF-α production but to inhibit LPS-dependent NO production under the same conditions (ZHANG and MORRISON 1993). As shown recently, macrophages express high levels of mRNA encoding the PT-sensitive G-protein subunit $G_i\alpha_2$, which is located on the plasma membrane and is associated with intracellular vesicles (XIE et al. 1993). In LPS-stimulated macrophages, $G_i\alpha_2$ was internalized and detected in association with vesicles and vacuoles (SWEET and HUME 1996). As PT mimics the cytoplasmic vacuolation induced by LPS (XIE et al. 1993), it was suggested that the role of PT-sensitive G-proteins in LPS-induced signal transduction should be reconsidered (SWEET and HUME 1996).

Transcription factors that appear to be involved in the activation of LPS-inducible genes include nuclear factor κB (NFκB), activator protein 1 (AP-1), the EtS family, Erg-1/2 and NF-IL-6. NFκB exists in the cytoplasm as homo- or heterodimers of a family of structurally related proteins. To date, five proteins belonging to the NFκB family have been identified: p65, cRel, RelB, p50/p105 and p52/p100. When present in the same cell, the different Rel-

related proteins can presumably complex with each other (May and Ghosh 1998). The dimeric complexes, of which p50/p65 is the most abundant, are normally present in the cytosol in an inactive form bound to an inhibitor protein, IκB. Phosphorylation of IκB induces dissociation of NFκB, followed by translocation of NFκB to the nucleus in an active form that binds to DNA (May and Ghosh 1998). IκB proteins are phosphorylated by the IκB kinase complex (IKK), which consists of two subunits, IKKα and IKKβ (Zandi et al. 1997).

Activation of NFκB plays an important role in the induction of various genes by LPS (Muller et al. 1993; Tebo et al. 1994). As LPS-mediated activation of NFκB also occurred in macrophages from C3H/HeJ (LPS-hyporesponsive) mice, albeit at higher LPS concentration, it has been suggested that NFκB activity alone is not sufficient for LPS activation (Ding et al. 1995). Another key component in LPS-inducible gene expression is AP-1 (Newell et al. 1994). AP-1 is a complex, DNA-binding protein composed of hetero- or homodimers of several proto-oncogenes, including c-jun, c-fos, junB and junD. The specificity of AP-1 for activating various genes depends on its composition and degree of phosphorylation (Brach et al. 1993). In the murine macrophage cell line J774, increases in junB and c-jun mRNAs were observed in response to LPS (Fujihara et al. 1993), and expression of c-fos was induced by LPS in murine macrophages (Collart et al. 1987).

Of the Ets transcription-factor family, two members (Ets and Elk-1) were demonstrated to be LPS-inducible in macrophages (Boulukos et al. 1990) and, recently, PU.1, another Ets-family member, was implicated in LPS signalling (Kominato et al. 1995; Lodie et al. 1997). Both Erg-2, a member of the early-growth response (*egr*) gene family (Coleman et al. 1992), and NF-IL-6 appear to be involved in the transcriptional activation of LPS-inducible genes in macrophages (Dendorfer et al. 1994).

F. Strategies for the Treatment of Gram-Negative Infections

Gram-negative sepsis represents a dramatic clinical picture associated with high morbidity and mortality (Nogare 1991; Bone 1993; Morrison et al. 1994). Despite considerable progress in the fields of modern antibiotic research and application and in intensive-care medicine, the high sepsis lethality was not lowered significantly during the last decades (Bone et al. 1989, 1990). Bacterial LPS constitutes a major factor responsible for pathological manifestations of gram-negative sepsis and its most dramatic outcome, septic shock. LPS is released in high amounts from bacteria, particularly in the course of antibiotic therapies, and the selective blockade of an overwhelming activation of different target cells would represent an important approach to the control of this severe disease.

Successful strategies for the treatment of gram-negative infections in general and the development of septic shock in particular should lead to the eradication of the causing agent and should interfere with LPS recognition, ideally at the very start of the LPS recognition pathway. In principle, each step of the endotoxic activation cascade (Fig. 2) could serve as a basis for the development of new therapy concepts (Levin et al. 1995). However, the early phases concerning the released bacterial toxin itself as the initial stimulus and its interaction with enhancing and inhibiting humoral factors (LBP, sCD14, BPI) and cell-bound receptors (mCD14, TLR-2) are of special interest. This concept is supported by the consideration that focussing on the early events of the septic cascade would lead to increased selectivity; therefore, fewer side effects would be caused by an antiseptic drug. In addition, an early therapeutic blockade (or even prophylactic medication) seems to be required from the clinician's point of view because, in practice, the development of septic shock is often difficult to predict (Wenzel et al. 1996). In this respect, non-toxic LPS, LPS partial structures and lipid-A analogs (Rietschel et al. 1996) that possess antagonizing activity appear to be promising candidates.

It is known from earlier studies that sublethal doses of endotoxin render the host temporarily refractory to subsequent LPS challenge, a phenomenon referred to as endotoxin tolerance (Freedman 1960; Rietschel et al. 1973; Greisman et al. 1979). Depending on the time of onset of the tolerance state, one can distinguish between an early- and a late-phase tolerance. Whereas the latter was shown to be mediated by O-specific antibodies and, thus, was specific for the inducing stimulus, the early-phase tolerance was mediated by the lipid-A component and was independent of the LPS serotype. The mechanisms of this early-phase tolerance are not well understood; however, it has been shown that post-translational regulation leads to a reduced TNF-α release after a second stimulus with endotoxin (Zuckerman et al. 1989). Additionally, several other genes encoding pro-inflammatory cytokines, such as IL-6, IL-1β and TNF-α, have been shown to become suppressed at the transcriptional level (Munoz et al. 1991a, 1991b; McCall et al. 1993). As outlined previously, the susceptibility of mice to endotoxins is determined by genetic and environmental factors (Freudenberg et al. 1998). This also holds true for other higher organisms, including humans. The gene locus involved in genetically determined hyposensitivity (Coutinho and Meo 1978; Rosenstreich 1985) has been identified, and the recent identification of its product (Du et al. 1998) may create additional strategies for the treatment and/or prevention of endotoxic shock.

I. Antibacterial Agents

Recently, evidence that the treatment of gram-negative bacteria with appropriate antibiotics may have an important impact on the activation of the immune response has been provided (Morrison 1998). It was observed that

the antibiotics moxolactam, gentamicin and chloramphenicol differ in their capacity to release LPS from bacteria. Similarly, it was observed that treatment of *P. aeruginosa* with imipenem resulted in the release of less endotoxin than treatment with ceftazidime, an effect attributed to the interaction of the antibiotics with different penicillin-binding proteins. These initial observations have gained support from several other studies and, more importantly, can be transferred to the in vivo situation, where imipenem treatment proved to be superior to ceftazidime in *Pseudomonas*-induced infections in rats. Imipenem treatment was accompanied by lower circulating TNF-α levels, probably due to lower amounts of released endotoxin. A recently conducted double-blind clinical trial confirmed these observations (Prins et al. 1995).

Since LPS is unique to gram-negative bacteria, it is considered as a potential target for the development of new types of antibiotics designed to inhibit LPS biosynthesis. Kdo is indispensable for the viability of gram-negative bacteria, and inhibitors of Kdo biosynthesis have been regarded as a potent new class of drugs (Unger 1981). Analogs that inhibit the activation of Kdo by CMP–Kdo synthetase prior to its incorporation into LPS (and, therefore, lead to the cessation of LPS biosynthesis) have been synthesized (Claesson et al. 1987; Goldman et al. 1987; Sarabia-Garcia et al. 1994). These compounds were reported to be bacteriostatic in vitro. Other synthetic compounds directed against another enzyme involved in Kdo biosynthesis, arabinose-5-phosphate isomerase, inhibited the enzyme but did not show antibacterial activity (Bigham et al. 1984).

Inhibitors of the early steps of LPS biosynthesis [such as the enzyme LpxC, which catalyzes the second step of lipid-A biosynthesis, i.e., the *N*-deacetylation of 3-*O*-acylated uridine diphosphate–*N*-acetyl-D-glucosamine (UDP–GlcNAc)] have been developed and exhibit good antibacterial activity against *E. coli* (Onishi et al. 1996). Unfortunately, these inhibitors were inactive against *Pseudomonas* strains, one of the major pathogens causing nosocomial infections and septic shock. Recently, the crystal structure of UDP–GlcNAc acyltransferase (LpxA, which is involved in the first step of lipid-A biosynthesis; Raetz 1996) was elucidated, providing a basis for the design of inhibitors of this enzyme.

A related conceivable strategy would employ inhibitors that interfere with later steps of early LPS-core biosynthesis, rendering bacteria unable to attach their protective *O*-antigens. Such bacteria would be cleared more efficiently by phagocytic cells and would show enhanced susceptibility to complement. In addition, cross-reactive epitopes may become more accessible for recognition by antibodies. Another example of defined interference with LPS biosynthesis was reported recently (Mamat et al. 1995). By the use of a trans-acting RNA derived from the *Acetobacter methanolicus* phage Acm1, it was possible to block LPS biosynthesis in several Enterobacteriaceae, such as *E. coli*, *S. enterica* and *Klebsiella pneumoniae*. The treatment resulted in the downregulation of O-specific-chain biosynthesis and the concomitant reduction of pathogenicity.

II. Antagonists of Endotoxic Effects

During investigations in which the nature of the interaction between lipid A and target cells was characterized in detail, we determined the binding of radioactively or fluorescently (fluorescein isothiocyanate) labeled LPS to the murine macrophage cell line J774.1 or to human peripheral monocytes. For both systems, saturable binding kinetics were obtained (KIRIKAE et al. 1993). The binding was completely inhibited by nanogram quantities of unlabeled R- or S-forms of LPS (Fig. 9). In further experiments, we examined defined partial structures of lipid A with respect to their ability to competitively inhibit binding of LPS. After cleavage of the fatty acids from the hydrophilic saccharide backbone, no inhibition (and, therefore, no binding to the receptor) could be detected. These compounds also did not induce cytokine production. Therefore, the hydrophobic region of lipid A seems to be important for binding to target cells and for biological activity. A partial structure of lipid A with four fatty acids could be identified. This was synthetic compound 406, which corresponded to the biosynthetic lipid-A precursor molecule from *E. coli* (lipid-A precursor Ia or lipid IVa; Fig. 5). This compound completely inhibited the binding of LPS (Fig. 10) and was not able to induce the release of pro-inflammatory cytokines from human peripheral monocytes (Fig. 10). These results suggest that the initial binding of LPS to target cells occurs via the *hydrophilic*, bisphosphorylated saccharide backbone (acyl groups also play an important but non-specific role) before additional structural characteristics of the *hydrophobic* region of lipid A initiate cell activation and the release of cytokines.

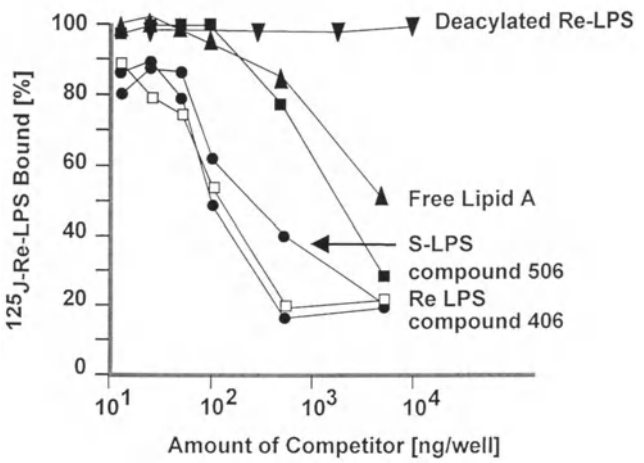


Fig. 9. Competitive inhibition of lipopolysaccharide (*LPS*) binding in the murine macrophage cell line J774.1. Compared with control, binding of ^{125}I-labeled Re LPS was inhibited by increasing concentrations of LPS, free lipid A or defined partial structures of Re LPS

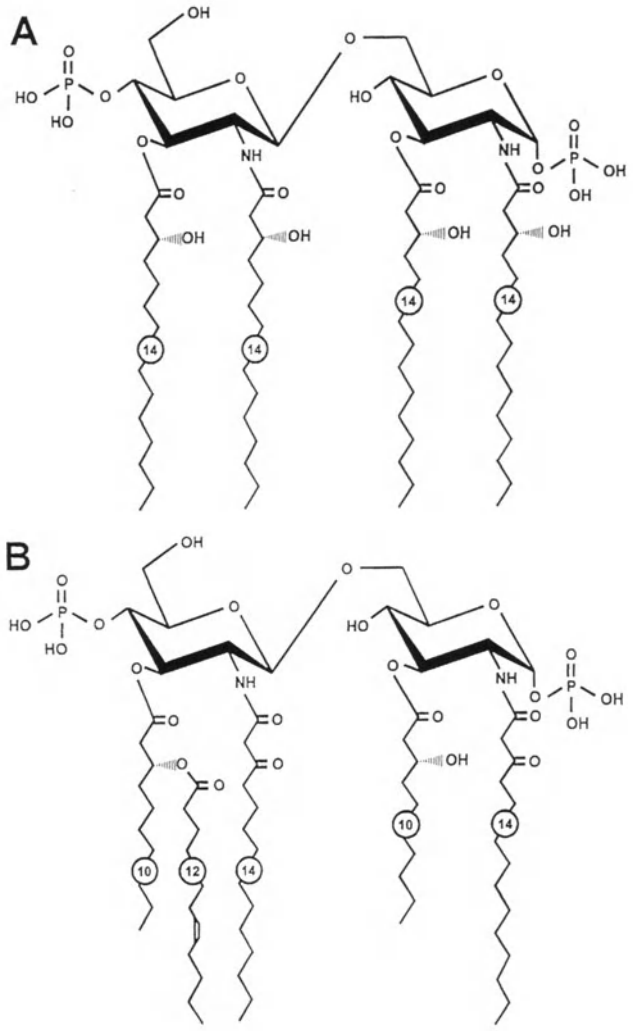


Fig. 10. Chemical structure of synthetic lipid-A partial-structure-compound 406 (**A**) and non-toxic lipid A from *Rhodobacter capsulatus* (**B**)

The segregation of lipid-A "binding" and lipid-A-mediated "activation" suggested that endotoxically inactive LPS or partial structures of LPS may bind to target cells without activating them and may, therefore, represent inhibitors of endotoxic effects. This assumption was confirmed by us (Loppnow et al. 1989a) and by other groups with respect to lipid-A precursors from *E. coli* (Kovach et al. 1990; Wang MH, et al. 1991) and *S. enterica* sv. Typhimurium (Golenbock et al. 1991; Lynn and Golenbock 1992). The tetra-acylated synthetic compound 406 inhibits the LPS-induced release of cytokines from

human monocytes specifically and in a dose-dependent manner (Loppnow et al. 1989a; Flad et al. 1993). This inhibition occurs at a very early stage of cytokine production; both the LPS-induced phosphorylation of proteins (Heine et al. 1995) and the specific synthesis of mRNA for IL-1 and TNF-α (Feist et al. 1992) are suppressed. Furthermore, in endothelial cells, the LPS-induced expression of intercellular adhesion molecule 1 (Schönbeck et al. 1994) and the synthesis of IL-6 were prevented by compound 406 (Loppnow et al. 1993). The tetra-acylated partial structure 406 of *E. coli* and non-toxic lipid-A preparations from the phototrophic bacteria *R. sphaeroides* (Takayama et al. 1989; Qureshi et al. 1991) and *R. capsulatus* (Fig. 10; Loppnow et al. 1990b, 1993) were also potent inhibitors of endotoxic effects. Recently, these observations resulted in the chemical synthesis of an antagonistic, penta-acylated analog based on lipid A from *R. capsulatus* (substance E5531; Christ et al. 1995). This molecule reduces endotoxin-caused lethality in mice and, together with antibiotic medication, is able to protect the animals from *E. coli*-induced lethal peritonitis. In a phase-I clinical study, substance E5531 showed its efficiency by inhibiting endotoxemic effects.

The mechanism underlying the inhibitory effect of lipid-A partial structures (such as compound 406) was intensively examined in different cell systems (Loppnow et al. 1989a; Kitchens et al. 1992; Lynn and Golenbock 1992; Flad et al. 1993; Kirikae et al. 1993, 1994; Heine et al. 1994; Kitchens and Munford 1995). We propose that these substances and active endotoxin directly compete for a surface-bound receptor. Our suggestion is supported by the fact that monokine production due to other stimuli, such as phorbol ester or lipopeptide, is not influenced by compound 406 (Loppnow et al. 1989a; Wang et al. 1991; Flad et al. 1993). In addition, the level of inhibition clearly depends on the amount of the antagonist and can be prevented by an excess of LPS. Finally, an antagonistic effect could be proven by a quantitative evaluation of the binding using Lineweaver/Burk plots (Heine et al. 1994). Based on these and other results (Kirikae et al. 1994), we conclude that inhibitory compounds, such as compound 406, lipid A from *R. sphaeroides* or substance E5531, block an LPS-specific binding site on the target cells and, thus, prevent binding and activation by endotoxins. CD14 could represent the putative membrane protein, because it is known that mCD14 can bind substance 406 and that the antagonistic effect of this lipid-A partial structure is abolished by an antibody against CD14 (Hailman et al. 1994). In contrast to this assumption, other studies using the cell line THP-1 suggest a non-antagonistic mechanism (Kitchens et al. 1992) that leads to the inhibition of a cellular response to LPS.

III. Neutralizing Antibodies Against Endotoxin

In addition to antagonistic molecules that block the activity of endotoxins at the level of target cells, substances that can directly neutralize the activity of released endotoxins have recently been discussed for use in the prophylaxis

and therapy of gram-negative septic shock. Among these molecules, immunoglobulins (Ig) represent classical therapeutic drugs that, for many reasons, appear particularly suitable. Antibodies against endotoxins can interrupt the activation cascade of gram-negative sepsis at a very early stage. In addition, they are distinguished by good host tolerance and a long half-life in the patient's body because of their high specificity and low immunogenicity. Finally, antibodies in complexes with their specific antigens may activate the complement system or bind to Fc receptors of phagocytes and, therefore, may contribute to an accelerated, non-inflammatory elimination of endotoxins. Due to these advantages, an immunological approach for the therapy of gram-negative sepsis has been pursued for a long time.

In principle, all structural regions of the LPS molecule exhibit immunogenic and antigenic properties. Thus, polyclonal (Saxen et al. 1986) and monoclonal (Kirkland and Ziegler 1984; Baumgartner et al. 1990) antibodies against determinants of the O-specific chain were protective in different endotoxin and infection models. However, the therapeutic benefit of these sera and antibodies are limited, because of the high serotype variability of the O-specific chain, especially among the clinically relevant bacteria *E. coli*, *K. pneumoniae* and *P. aeruginosa*. Compared with the O-specific chain, the lipid-A component of many pathogenic microorganisms is an extraordinarily conserved region (Zähringer et al. 1994). In addition, the center of the endotoxic activity is localized in this LPS domain (Galanos et al. 1985) which, therefore, seems to be a target structure for the production of neutralizing antibodies with broad cross-reactivities to different pathogenic, gram-negative bacteria. Interestingly, antibody specificities against free lipid A could hardly be detected in the sera of experimental animals immunized with R- and S-form bacteria. High-titer antisera (Galanos et al. 1984) and monoclonal antibodies (Dunn et al. 1986; Bogard et al. 1987) could, however, be generated with free lipid A as the immunogen and were characterized (together with different lipid-A preparations and partial structures) according to their specificities (Brade et al. 1988, 1993; Kuhn et al. 1992). These antibodies showed cross-reactivity with different lipid-A molecules that had been liberated from their polysaccharide portions (Galanos et al. 1984; Brade et al. 1988). The epitopes of all investigated antibodies were determined to be located in the hydrophilic saccharide backbone. However, no reactivity was found when lipid A was substituted for the saccharide portion of the core region. The rationale for this seems to be that the primary hydroxyl group of the non-reducing D-glucosamine residue (GlcN-II in Fig. 4) serves as the epitope for lipid-A-specific antibodies and, as such, is not freely accessible in LPS when substituted with Kdo. Thus, lipid A has to be regarded as a neoantigen, and cross-reactivity between lipid A and LPS is unlikely (Brade et al. 1997). Therefore, during a septic episode, a protective effect of lipid-A antibodies is unlikely to be successful, and clinical studies have indeed failed to show beneficial effects (Wenzel et al. 1991; Baumgartner and Glauser 1993).

The chemical analysis of core oligosaccharides of numerous LPS showed that, in many strains and species of microorganisms, this region contains con-

served structural elements (Holst and Brade 1992). Many clinically relevant isolates from the family of the Enterobacteriaceae share common characteristics of the inner-core region and its adjoining segments (Fig. 3). By immunizing NZB-mice with LPS from *E. coli* mutants [with different core types (R1–R4) as immunogens], we have been able to produce monoclonal antibodies that show broad cross-reactivity with R- and S-forms of LPS from *E. coli*, *S. enterica* and *Shigella* (Di Padova et al. 1993a, 1993b). In addition, these antibodies also exhibited anti-endotoxic properties. One of these monoclonal antibodies, termed WN1-222-5 (IgG2a), was able to offer potent protection against the lethal effect of different endotoxins in mice and to reduce dramatically the fever response of rabbits after LPS application (Bhat et al. 1994). In addition, a clear improvement in the survival rate was observed in infection experiments with mice (Bailat et al. 1997). For one potential application in patients, a human chimeric IgG1 isotype (SDZ-219-800) was finally created from WN1-222-5 (Di Padova et al. 1994) to achieve a better tolerance and half-life of the antibody. Further experiments also proved the binding to all clinical isolates from *E. coli*, *S. enterica* and some other Enterobacteriaceae. In addition, SDZ-219-800 could efficiently neutralize these endotoxins in different experimental systems in vitro and in vivo (Di Padova et al. 1993a, 1993b).

The antibodies WN1-222-5 and SDZ-219-800 show no reactivity with free lipid A. The LPS epitope, which is also accessible for binding by these antibodies in the presence of the O-specific chain, includes structural elements of the inner- and outer-core regions (Fig. 3). Therefore, no direct binding to the endotoxic center of LPS (i.e., lipid A) is necessary for the neutralizing effect of the antibodies. Steric effects of the bound antibody (or a conformational change of LPS induced by the binding of the antibody) could prevent the binding of endotoxins to LBP or CD14 (Pollack et al. 1997). In addition, an increased elimination of LPS–antibody complexes by Fc receptors and/or the complement system is conceivable for the neutralizing effect in vivo. The mechanisms underlying the actions of these antibodies have been studied recently in vitro. WN1-222-5 was shown to inhibit the association of LPS–LBP complexes with mCD14 on monocytes (Pollack et al. 1997).

Therefore, cross-reactive monoclonal antibodies against the core oligosaccharide of LPS can be regarded as potent neutralizing agents of endotoxin. In the near future, further development and characterization of these antibodies may make it possible to create an innovative immunotherapy for patients suffering from gram-negative sepsis.

G. Final Remarks

Since the discovery of endotoxins more than a century ago, considerable progress has been made towards the elucidation of structure–activity relationships of endotoxins; this was a challenging and difficult undertaking due to the chemical nature, high biological potency and pleiotropic biological

effects of endotoxins. Increased knowledge of the chemical structure of endotoxins together with advances in biotechnology and cell biology led to the identification of key players involved in endotoxin action, and promising targets for the development of new antibacterial drugs and the treatment of gram-negative sepsis have emerged. However, further characterization of endotoxic action (on the molecular/atomic level) of isolated LPS and LPS associated with bacteria will be required to reach the final goal, i.e., the treatment of patients suffering from the lethal actions of endotoxins and severe infections with gram-negative bacteria. Equally important will be an improved and detailed knowledge of LPS biosynthesis. The most effective approach to preventive and therapeutic treatment of endotoxin-based diseases may require a combination of different strategies that interfere with LPS biosynthesis and endotoxin action at different steps of the cellular-activation cascade. The transfer of our improved knowledge into clinical medicine remains a challenging task and will require basic research and applied clinical medicine.

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CHAPTER 28

Translocated Toxins and Modulins of *Yersinia*

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A. Introduction

The bacterial genus *Yersinia* includes three human pathogenic species: *Y. pestis* is the causative agent of plague, which is transmitted by infected rat fleas. *Y. pseudotuberculosis* and *Y. enterocolitica* are well-known pathogens causing food-borne enteric diseases. These microorganisms can be visualised microscopically as gram-negative rods. Moreover, they have typical features in common with other species of Enterobacteriaceae: the cell envelope is composed of a cytoplasmic membrane, a peptidoglycan/murein sacculus and an outer membrane containing the typical lipopolysaccharides (endotoxins), porins and other components. The pathogenicity of *Yersinia* spp. is controlled by chromosomal and extrachromosomal determinants. *Y. enterocolitica* and *Y. pseudotuberculosis* carry the chromosomal gene *inv*, which codes for a 95-kDa outer-membrane protein (invasin, Inv) with the capacity to interact specifically with β1 integrins and trigger uptake (Isberg and Leung 1990). This feature of Inv led initially to the assumption that Yersiniae are intracellular pathogens. However, functions of the *Yersinia* virulence plasmid (pYV, ~70 kb) that are involved in inhibiting phagocytic processes and favouring extracellular survival and replication in host tissue were later recognised. Interestingly, all three pathogenic Yersiniae harbour pYV and, thus, express common pathogenic factors. The general organisation of the pYV plasmid has been described elsewhere (Iriarte and Cornelis 1998b). The virulence genes of Yersiniae are coordinately regulated by a network, a so-called virulon, which can be triggered by environmental signals (pH, Ca^{2+}, temperature, cell receptors etc.). Upon ingestion of Yersiniae, the bacteria reach the intestinal lumen (where *inv* is upregulated), but the majority of the virulence genes of pYV are downregulated. This expression mode probably enables Yersiniae to interact with β1-integrins of M-cells and to trigger intracellular uptake and translocation into the subepithelium of Peyer's patches. Within this environment, the virulence genes on the pYV plasmid are upregulated, which allows Yersiniae to grow extracellularly by impeding the primary defense of the host: resistance to complement lysis and defensins, inhibition of oxidative burst of phagocytes, inhibition of the release of pro-inflammatory cytokines and phagocytosis and induction of apoptosis in macrophages.

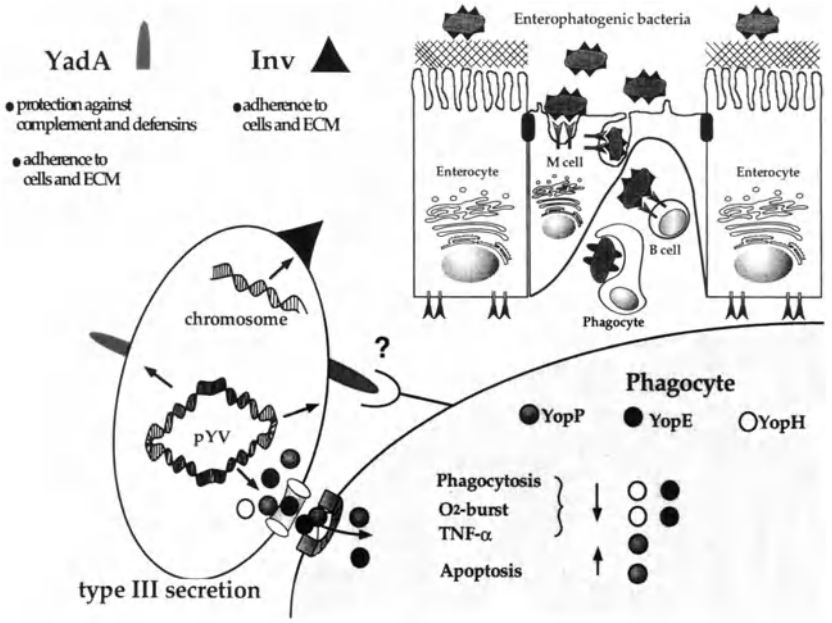

Fig. 1. Model showing the mechanisms by which *Yersinia* invades intestinal cells and resists uptake by phagocytes. *YadA Yersinia* adhesin, *Inv Yersinia* invasin, *ECM* extracellular matrix, *pYV Yersinia* virulence plasmid

During the last decade, it became evident that the *Yersinia* pYV plasmid carries gene clusters encoding for (1) a protein type-III secretion/translocation apparatus, (2) a set of at least six effector proteins (*Yersinia* outer proteins, Yops), which are translocated through a "translocation needle" from the cell-adhering bacterium into the cytoplasm of the host cell, (3) regulators for gene expression and Yop secretion/translocation and (4) a *Yersinia* adhesin (YadA), which presents a lollipop-shaped surface projection with multiple functions (protection against complement and defensin lysis, binding to extracellular-matrix proteins and adherence to diverse cell types, including professional phagocytes). The current opinion on the way in which Yersiniae can invade intestinal cells and resist uptake is schematically depicted in Fig. 1.

B. *Yersinia* Protein Type-III Secretion/Translocation System

I. Virulence Plasmid pYV

According to their presumed function, the secreted Yops encoded by the pYV plasmid have been divided up into effectors, translocators and regulators (CORNELIS and WOLF WATZ 1997). The effector Yops include YopH, YopE,

YopM, YopT, YopO/YpkA and YopP/YopJ. All of the effector Yops have been demonstrated to be translocated into the target cell, most of them (except YopM) have been shown alter some kind of cell function and none of them seem to be required for secretion or translocation of any other Yop.

The translocator Yops include YopB, YopD and presumably LcrV and YopK (*Y. pseudotuberculosis*)/YopQ (*Y. enterocolitica*). YopB and YopD are individually required for translocation of all the other Yops. YopB is thought to create a pore of about 1.2–3.5 nm diameter in the eukaryotic cell membrane, through which Yops are translocated (Hakansson et al. 1996a). YopD is an essential component of the translocation apparatus but also acts as a negative regulator of Yop expression and translocates into the cytosol of target cells (Francis and Wolf Watz 1998; Lee et al. 1998). Thus, YopD might integrate Yop expression and translocation. YopN likely regulates translocation after bacterium target cell contact has taken place. This is based on observations that YopN mutants release large amounts of Yops into the extracellular medium (even in the presence of Ca^{2+}), yet are still able to inject Yops into the target cell (Rosquist et al. 1994).

The ~22 proteins of the type-III secretion system (Ysc) are encoded by four loci on the pYV plasmid called virA, virB, virC and virG/virF. Mutants in most of these proteins are defective in secretion. Through sequence homologies, functional and mutational studies and immunolocalisation, a presumptive model of how the secretion apparatus might be organised was proposed. Details of this model can be read elsewhere (Cornelis and Wolf Watz 1997; Hueck 1998). A strongly simplified model of the secretion/translocation apparatus is included in Fig. 5.

The *Yersinia* adherence protein YadA is also encoded by the pYV plasmid and comprises a 200-kDa oligomer of individual 45-kDa subunits. Distinct domains of YadA have been implicated in a variety of adhesion phenomena attributed to virulent Yersiniae. For example, residues 29–81 are necessary for interaction with neutrophils (Roggenkamp et al. 1996), residues 81–100 comprise a hydrophobic domain that binds to collagen and mediates *Yersinia* autoagglutination (Skurnik and Wolf Watz 1984), and amino acids 445–455 constitute a hydrophobic stretch involved in the anchoring of YadA into the outer membrane (Tamm et al. 1993).

II. Regulation of Yop Expression, Secretion and Translocation

Virulent *Yersinia* spp. growing at 27°C reduce the growth rate when shifted to 37°C and Ca^{2+}-deprived medium. The effects of the increase in temperature and Ca^{2+} deprivation were found to be dependent on the pYV plasmid, which allows expression and release of Yops into the surrounding medium under these conditions (Heesemann et al. 1984, 1986a; Heesemann 1986b). Figure 2 depicts a Coomassie-stained sodium dodecyl sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) gel of Yops released by *Y. enterocolitica* (0:8) by Ca^{2+} deprivation.

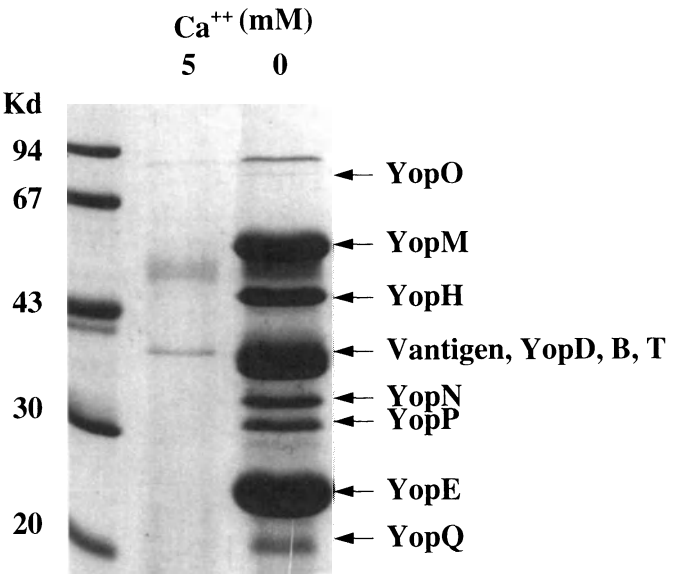

Fig. 2. Yops released by *Yersinia enterocolitica*. *Y. enterocolitica* serotype O8 growing in high-Ca^{2+} medium (5 mM Ca^{2+}) was shifted to Ca^{2+}-deprived medium. Released proteins in the supernatant were run on sodium dodecyl sulfate polyacrylamide-gel electrophoresis and stained with Coomassie

The role that Ca^{2+} deprivation plays for Yop expression and secretion is not clear, because the free extracellular Ca^{2+} concentration is ~1 mM in interstitial fluid and blood and, thus, would not allow Yop expression in vivo. Potentially, at the bacterium–target cell interface, a compartment with low Ca^{2+} concentration is created, and a Ca^{2+} sensor (for example YopN) located at this interface could transmit a signal into the cell (FORSBERG et al. 1991). In addition to temperature and Ca^{2+}, another regulation mechanism exists that inhibits Yop expression if the secretion apparatus has not been "opened" by bacterium–target cell contact. The current idea is that this suppressor is LcrQ (*Y. pseudotuberculosis*)/YscM (*Y. enterocolitica*), a protein encoded by the pYV plasmid secreted via the type-III apparatus. Secretion lowers the concentration of LcrQ/YscM in the bacterial cytoplasm and, therefore, releases the block imposed on Yop gene expression (PETTERSON et al. 1996).

By using *Y. enterocolitica* producing truncated YopE and YopH proteins fused to calmodulin-dependent adenylate cyclase from *Bordetella pertussis*, the N-terminal 15 and 17 amino acid residues, respectively, were identified as the minimal domains required for secretion. Similarly, the N-terminal 50 and 71 amino acids were found to be required for translocation of YopE and YopH, respectively (SORY et al. 1995). As proposed recently (CORNELIS and WOLF WATZ 1997), the term "secretion" is used to describe export of Yops through the inner and outer bacterial membrane, whereas translocation is used to

describe transport through the eukaryotic cell membrane. Secretion domains within the first 10–20 N-terminal amino acids and translocation domains within the first 100 N-terminal amino acids were also identified in YopM, YopP and YopT (Boland et al. 1996; Iriarte et al. 1998a, 1998b). Surprisingly, the N-terminal amino acids required for Yop secretion do not have properties of signal peptides and are not cleaved off during secretion. Moreover, there is no sequence homology between the signal peptides of the different Yops, and neither point mutations nor frameshift mutations in the Yop gene, which caused an altered N-terminal amino acid sequence, abolished secretion. This led to the suggestion that messenger RNA structure (rather than peptide sequence) constitutes the secretion signal (Andersson and Schneewind 1998).

The amino acids of YopE that constitute the translocation signal also mediate binding to a protein designated specific YopE chaperone (SycE). A chaperone function of SycE has been proposed on the basis of experiments showing that deletion of the SycE gene, which is located close to the YopE gene on the pYV plasmid, essentially abolished secretion but not intracellular accumulation of YopE (Wattiau et al. 1993). In another report, a SycE mutant was found to degrade YopE much more rapidly than the wild type (Frithz-Lindsten et al. 1995). Sycs have also been identified for YopH (SycH), YopT (SycT) and YopB, D (SycD; Wattiau et al. 1994; Iriarte et al. 1998a). Recently it was demonstrated that both the N-terminal secretion domain and the SycE-binding domain are necessary for targeting of YopE into the cytosol of HeLa cells (Lee et al. 1998). The current concept is that SycE, SycH and SycT bind to their nascent Yop partners and protect them from proteolytic degradation. It is not clear at present whether Sycs also have folding/refolding activities and/or regulate association with the secretion apparatus. A schematic diagram of the Yop secretion, translocation and functional domains (as identified by different investigators) is depicted in Fig. 3.

When the "translocator" YopD was mutated in a *Y. pseudotuberculosis* strain, YopE was secreted normally but was not translocated. Instead, it showed a patchy, extracellular distribution, which was confined to the contact zone of the bacterium and target cell. This result suggested that Yop secretion/translocation is "polarised" in the sense that it occurs only at the zone where the bacterium and target cell have direct contact (Rosquist et al. 1994).

The elucidation of the molecular mechanisms of Yop secretion and translocation has made it possible to design *Yersinia* strains translocating a specific protein of interest into eukaryotic target cells. Translocation of heterologous proteins composed of the YopE translocation/secretion domain fused to adenylate cyclase, neomycin transferase or green fluorescent protein has already been reported (Sory et al. 1995; Jacobi et al. 1998; Lee et al. 1998). Considering that the *Yersinia* type-III system could be engineered to translocate single effector Yops (Roggenkamp et al. 1995), it can anticipated that any single protein of interest can be introduced into target cells without

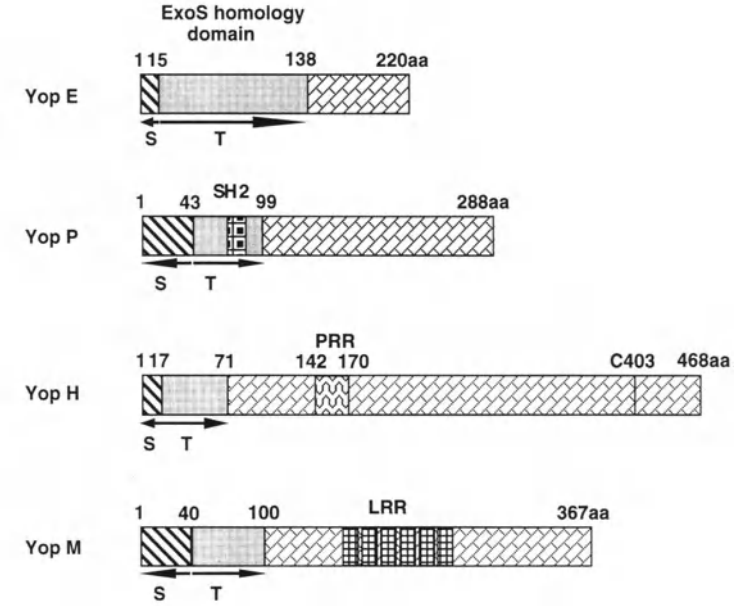

Fig. 3. Translocation, secretion and functional domains of different Yops. *S* secretion domain, *T* translocation domain, *SH2* Src homology-2 domain, *aa* amino acids, *LRR* leucine-rich repeats, *PRR* proline-rich repeats, *ExoS* exoenzyme S from *Pseudomonas aeruginosa*

interference by other Yops. Clearly, some proteins essential for Yop translocation, such as YopD and YopN, will inadvertently be co-introduced into cells.

C. Translocated Toxins and Modulins of *Yersinia* (Effector Yops)

I. YopH, a Highly Active Tyrosine Phosphatase

YopH is a 51-kDa protein essential for virulence in the mouse model (Bölin and Wolf Watz 1988). It has been shown to represent a protein tyrosine phosphatase (PTPase) with sequence homology to eukaryotic PTPases (Guan and Dixon 1990). In fact, YopH is among the most active PTPases known (Zhang et al. 1992). The catalytic domain of YopH is located in the C-terminal half of the protein and includes, as in other tyrosine phosphatases, an essential cysteine residue (Cys 403) implicated in the formation of a reaction intermediate during phosphate hydrolysis (Zhang and Dixon 1993). Mutation of Cys403 to Ser (YopHC403S) or Ala (YopHC403A) in YopH was shown to abrogate PTPase activity and produced mutants that trapped the putative cellular substrates of YopH (Bliska et al. 1992). In the central part of YopH, a poly-proline

region that appears to bind to Src homology 3 (SH3) domains of host cell proteins is located (Bliska 1996). Crystallisation of purified YopH revealed that it consists of an eight-stranded β-sheet and seven α-helixes, which together form the substrate-binding cleft. The region encompassing amino acids 403–410 is located at the base of the cleft and constitutes the phosphate-binding loop (Stuckey et al. 1994).

Wild-type strains of *Y. pseudotuberculosis* could resist phagocytosis by macrophages, but resistance was greatly reduced in strains depleted of the YopH gene. In a double mutant depleted of YopH and YopE, phagocytosis resistance was completely abolished, as in the avirulent, plasmid-cured *Y. pseudotuberculosis* strain, which does not produce any Yops (Rosquist et al. 1988; Fallmann et al. 1995). Similarly, phagocytosis of complement opsonised *Y. enterocolitica*, and the subsequent oxidative burst in neutrophils could be suppressed by about 50% by a *Y. enterocolitica* mutant that expressed and translocated only YopH and could be completely abolished by a mutant expressing both YopH and YopE. (Ruckdeschel et al. 1996). In addition, invasin-dependent uptake of *Y. pseudotuberculosis* by HeLa cells was suppressed by a multiple Yop mutant (MYM) defective in expression of YadA, YopT, YopE, YopH, YopM and YpkA that was complemented with the YopH gene, but not by a MYM that was complemented with the YopHC403S mutant (Persson et al. 1997). These reports suggested that YopH inhibits uptake of *Yersinia* spp. via phagocytic and non-phagocytic mechanisms, presumably by interference with tyrosine phosphorylation events associated with the uptake process.

Infection of HeLa cells and J774 macrophages with wild-type *Y. pseudotuberculosis* strains caused tyrosine dephosphorylation of several proteins, including proteins between 120 kDa and 130 kDa and one protein of 68 kDa (Bliska et al. 1992; Black and Bliska 1997; Persson et al. 1997). Dephosphorylation of these proteins was abolished in YopH-negative mutants, whereas *Y. pseudotuberculosis* translocating the substrate-trapping mutant YopHC403S caused hyperphosphorylation of the 120–130-kDa proteins but not the 68-kDa protein. Thus the substrate-trapping mutant YopHC403S presumably binds to phosphotyrosyl residues and protects these sites from endogenous phosphatases, as was also found for "cysteine" mutants of eukaryotic PTPases, such as SHP-1-C453S (Plas et al. 1996). Not all tyrosine-phosphorylated proteins in HeLa cells were dephosphorylated by YopH, indicating that this PTPase displays substrate specificity, as do its eukaryotic counterparts. Several lines of evidence (Black and Bliska 1997; Persson et al. 1997) suggested that the Crk-associated tyrosine kinase substrate $p130^{Cas}$ (Cas) is the major YopH substrate in HeLa cells. (1) Cas is dephosphorylated by wild-type *Y. pseudotuberculosis*, and its dephosphorylation is prevented in YopH "knock out" Yersiniae, whereas it is hyperphosphorylated by Yersiniae producing YopHC403S. (2) YopHC403A co-localises with Cas and Fak in peripheral focal adhesions in HeLa cells, and these adhesions are dissolved by wild-type Yersiniae. (3) Using an overlay assay, glutathione *S*-

transferase–YopHC403A was found to directly bind to tyrosine-phosphorylated (but not dephosphorylated) Cas or to the focal adhesion kinase Fak. Supporting the notion that the Cas is an important YopH substrate, Cas contains 15 tyrosine-phosphorylation motifs (YXXP), at least ten of which conform to an optimal substrate-recognition sequence for YopH (Sakai et al. 1994; Zhang and Dixon 1994). Hence, the 120–130-kDa proteins dephosphorylated by YopH in HeLa cells presumably mostly represent Cas, containing different numbers of phosphorylated tyrosine residues.

Although Fak is dephosphorylated by YopH in intact cells and YopHC403S is found in Fak immunoprecipitates, dephosphorylation of Fak is much slower than that of Cas, and YopHC403S does not bind directly to Fak in the overlay assay (Black and Bliska 1997). This indicates that dephosphorylation of Fak might be rather indirect, potentially due to dephosphorylation of Cas in the vicinity of Fak. If Cas and Fak are indeed the major targets of YopH, the pathophysiological significance of this needs to be determined. Cas is considered a scaffold protein, because it contains various SH2-binding phosphotyrosyl motifs predicted to interact with Crk and tensin, a SH3 domain that was shown to interact with Fak and binding sites for the SH2 and SH3 domains of Src (Nakamoto et al. 1996). Cas and Fak get tyrosine phosphorylated after ligation of β1-integrins and co-localise with vinculin and paxillin in focal adhesions of fibroblasts (Nojima et al. 1996). Src and Fak also co-precipitate with Cas and seem to somehow control Cas phosphorylation (Sakai et al. 1994). Thus, Cas and Fak regulate integrin signalling via cell adhesion complexes at the cytoplasmic face of the plasma membrane and, therefore, could influence cell growth, differentiation and anchoring. Clearly, binding of invasin to β1-integrins and the subsequent signalling events are crucial for uptake of Yersiniae – at least those Yersiniae that do not express YadA – into non-phagocytic cells and for phagocytosis of non-opsonised Yersiniae (Fallman et al. 1995; Persson et al. 1997). These uptake mechanisms are also dependent on tyrosine kinase activity, and Cas and Fak are tyrosine phosphorylated during uptake of Yersiniae (Black and Bliska 1997; Persson et al. 1997). However, the requirement for Cas or Fak tyrosine phosphorylation in uptake or phagocytosis has not been demonstrated conclusively (for example, in Fak or Cas "knockout models"). In fact, the focal adhesion sites to which YopHC403S and Cas localised in HeLa cells did not co-localise with the sites to which Yersiniae were adhering (Black and Bliska 1997). Therefore, an important role of Cas and Fak dephosphorylation by YopH might be to block migration, cell–cell interaction and release of proteases and cytokines in macrophages, because these processes are known to be dependent on intact focal adhesions (Juliano and Haskill 1993; Lin et al. 1994). Further studies should be designed to identify additional targets of YopH in macrophages and antigen-presenting cells and to test the potential role of Cas in cell functions normally subverted by *Yersinia* spp. It would also be interesting to find out whether YopH is able to act on M-cells.

II. YopE, an Actin-Disrupting Cytotoxin

YopE is a 23-kDa protein required for full virulence in a mouse model of *Y. pseudotuberculosis* infection (Forsberg and Wolf-Watz 1988) and is a potent cytoskeleton disrupting toxin when introduced into tissue-culture cells (Rosquist et al. 1990, 1991). A truncated form of YopE lacking the C-terminal third of the protein is still secreted into the culture medium but does not induce a cytotoxic effect, indicating that the C-terminus contains the active domain of YopE (Rosquist et al. 1990). YopE has sequence homology to the N-terminal, non-catalytic region of the adenosine diphosphate (ADP)–ribosyltransferase exoenzyme S (ExoS) from *P. aeruginosa* (Kulich et al. 1994). Since ExoS ADP–ribosylates Ras and other unidentified, small guanosine triphosphate (GTP)ases of the Ras family (Coburn et al. 1989), it was speculated that YopE might affect the cytoskeleton through modification of Ras-like GTPases of the Rho family. Rho-GTPases are central regulators of actin in all cell types, and a number of bacterial toxins can activate or inactivate these GTPases. Interestingly, Rho-inactivating toxins like C3 transferase from *Clostridium botulinum* and toxin A from *C. difficile* cause cell rounding and actin-cytoskeleton disruption, as does YopE (see chapters by Just and Aktories). Recently, a *Salmonella* effector protein translocated through a type-III secretion apparatus was shown to stimulate nucleotide exchange and activate Rho-GTPases, and thus induced downstream actin rearrangements (Hardt et al. 1998). This work showed for the first time that a translocated bacterial effector can engage Rho-signalling pathways and should stimulate further efforts to relate Yops to Rho-GTPase-signalling events.

The effect of YopE on tissue-culture cells has been investigated mainly with *Y. pseudotuberculosis* strains mutated for YopE (Rosquist et al. 1990, 1991, 1994). In HeLa cells, wild-type *Y. pseudotuberculosis* induces a cytotoxic effect characterised by rounding up of the cells and disruption of the actin cytoskeleton, whereas micotubuli and intermediate filaments remain intact. This cytotoxic effect was abolished in YopE mutants (Rosquist et al. 1991). Interestingly, Yersiniae expressing the substrate-trapping mutant YopHC403S stabilised focal adhesion towards the action of YopE (Black and Bliska 1997). YopE clearly contributes to phagocytosis resistance of *Yersinia* spp. towards macrophages and neutrophils, because a double YopE/H mutant was significantly better phagocytosed by macrophages than the single YopH mutant, and a *Y. enterocolitica* mutant engineered to translocate only YopE and YopH suppressed phagocytosis and the oxidative burst in neutrophils significantly better than the *Yersinia* strain expressing only YopH (Ruckdeschel et al. 1996). When the ability of *Yersinia* Yops to block invasion of other pathogens was tested, YopE prevented invasion of *Listeria monocytogenes*, *Edwardsiella tarda* and *Shigella flexneri*, whereas YopH only prevented invasion of Listeriae. *Salmonella* invasion was not blocked by any Yop (Mescas et al. 1998). These studies, relying solely on recombinant *Yersinia* translocation

systems, suggest that YopE interrupts specific signal pathways associated with bacterial invasion and phagocytosis that only partially overlap the signal pathways interrupted by YopH. In one study, HeLa cells were overlaid with secreted *Yersinia* proteins or a lysate from *E. coli* expressing YopE and then agitated in the presence of glass beads (Rosquist et al. 1991). This method produced a cytotoxic effect in ~20% of cells "loaded" with proteins released from wild-type *Y. pseudotuberculosis* or *E. coli* expressing YopE but not with proteins from the YopE-mutant Yersiniae. In our study, when we microinjected purified His-tagged YopE protein from *Y. enterocolitica* into HeLa cells, no cytotoxic effect was obvious, whereas microinjected YopH protein disrupted focal adhesions and the actin cytoskeleton (Aepfelbacher et al., unpublished results). Therefore, it remains to be investigated whether purified YopE protein or a YopE-expression plasmid introduced into cells is able to produce an effect by itself, or whether it needs another Yop or some kind of modification acquired during secretion or translocation.

III. YopP, Modulator of Multiple Signal Pathways Leading to Apoptosis and Cytokine Suppression

YopP (*Y. enterocolitica*) and the highly homologous YopJ (*Y. pseudotuberculosis*) are 32.5-kDa proteins that, so far, have not been shown to be required for virulence in mouse models (Galyov et al. 1994). The secretion and translocation domains of YopP have been mapped to the first 43 and 99 amino acids, respectively (Iriarte et al. 1998b). Four regions of YopP and YopJ have sequence homology to the avirulence protein AvrRxv from *Xanthomonas campestris* pathovar vesicatoria, a plant cell pathogen that induces death in the infected cells, obviously through its intracellular injection via a type-III secretion pathway (Whalen et al. 1993; Mittler and Lam 1996). Recently, AvrA of *Salmonella enterica*, a protein secreted through a type-III secretion apparatus, was also shown to contain significant homology to the YopP/YopJ and AvrRxv proteins. AvrA, AvrRxv and YopJ/YopP might constitute a new family of intracellularly injected bacterial proteins that induce cell death in their hosts (Hardt and Galan 1997). These proteins also contain a region that is similar to motif I of the SH2 domains of $p56^{lck}$, Syk and Shc (Koch et al. 1991). Within this region, a conserved D/E–X–E motif is found which, when mutated to NAQ in YopJ (D53N-V54A-E55Q), abrogates YopJ activity (see details below).

Reports by three different groups suggested that YopP/YopJ is involved in the induction of apoptosis in *Yersinia*-infected mouse macrophage cell lines RAW264.7 and J774, or in mouse bone-marrow-derived macrophages (Mills et al. 1997; Monack et al. 1997; Ruckdeschel et al. 1997). These papers demonstrated that wild-type strains of *Y. enterocolitica* or *Y. pseudotuberculosis* produced typical signs of apoptosis, such as membrane blebbing, DNA degradation and nuclear fragmentation. The first signs of apoptosis were seen after 1 h of infection and, after 4–8 h, most macrophages were killed. *Y.*

enterocolitica or *Y. pseudotuberculosis* strains unable to express YopP/YopJ did not induce apoptosis whereas, in *Y. enterocolitica* mutants overexpressing YopP, apoptosis was enhanced (MILLS et al. 1997; MONACK et al. 1997).

An avirulent, plasmid-cured strain of *Y. enterocolitica* and *E. coli* lipopolysaccharide induced strong and continuous activation of nuclear factor (NF)-κB in J774 macrophages (RUCKDESCHEL et al. 1998). In contrast, interaction of wild-type Yersiniae with J774 macrophages caused a small and transient nuclear translocation of NF-κB followed by a refractiveness to activation of NF-κB (RUCKDESCHEL et al. 1998). These data suggested that a Yop was involved in downregulation of NF-κB activity. Indeed, a *Y. enterocolitica* mutant in the YopO, YopP-operon largely restored the persistent activation of NF-κB and, in HeLa cells infected with a *Y. pseudotuberculosis* YopJ mutant, NF-κB activation was completely restored (RUCKDESCHEL et al. 1998; SCHESSER et al. 1998). NF-κB is a heterodimer of two proteins (p50/p60); it is sequestered and kept inactive in the cytosol by the inhibitory proteins IκB-α or IκB-β. NF-κB gets activated when the IκB inhibitors are phosphorylated by IκB kinase and subsequently degraded through the ubiquitin–proteasome pathway (BALDWIN 1996). A careful analysis revealed that the ability of *Y. enterocolitica* to inhibit activation of NF-κB in J774 cells was due to the prevention of degradation of IκB-α and IκB-β at later times (30–120 min) after infection (RUCKDESCHEL et al. 1998). Consistent with the involvement of YopP/YopJ in reducing NF-κB activation, the YopJ mutant of *Y. pseudotuberculosis* could not prevent degradation of IκB in HeLa cells (SCHESSER et al. 1998). These reports suggest that YopP/YopJ of Yersiniae are involved in silencing NF-κB by inhibiting the phosphorylation and degradation of the IκB proteins in macrophages and epithelial cells.

Y. enterocolitica infection of J774 macrophages also led to a rapid tyrosine phosphorylation of mitogen-activated-protein (MAP) kinases 1 and 2 [extracellular-signal-regulated protein kinases (ERKs) 1 and 2], p38 and c-Jun N-terminal kinase (JNK), which was followed by a prolonged dephosphorylation of p38 and ERK1/2 (RUCKDESCHEL et al. 1997). The dephosphorylation of the MAP kinases coincided with suppressed in vitro phosphorylation of the transcription factors Elk-1, activating transcription factor 2 (ATF2), and c-Jun, confirming that MAP kinases were inhibited. The non-virulent *Yersinia* strain and a strain that expressed the *Yersinia* secretion and translocation machinery but no effector Yops were unable to induce dephosphorylation of ERK1/2 and p38 or to suppress phosphorylation of the transcription factors, indicating the requirement for one or more translocated Yop effector. One report confirmed inactivation of p38 and JNK in macrophages by *Y. pseudotuberculosis* and, furthermore, showed that a YopJ mutant was unable to downregulate p38 and JNK, whereas the downregulating activity was restored when the YopJ mutant was complemented with YopJ in trans (PALMER et al. 1998).

Apoptosis is tightly controlled by different pro-apoptotic and anti-apoptotic signal pathways that can positively and negatively influence each other and thus constitute a complex regulatory network. Not surprisingly, YopP

was implicated in downregulation of several of these signalling pathways, including mitogen-activated protein kinases (p38, JNK and ERKs 1 and 2) and NF-κB. Since activation of NFκ-B has been reported to protect against apoptosis, the YopP/YopJ-dependent downregulation of NF-κB activity could trigger the apoptosis observed in *Yersinia*-infected macrophages. In fact, when J774 cells were treated with the proteasome inhibitor MG-132 to inhibit NF-κB activation, even plasmid-cured Yersiniae (which produce no Yops) were able to induce apoptosis (RUCKDESCHEL et al. 1998). The central signal molecules initiating and promoting apoptosis are a number of cysteine proteases called caspases, which cleave structural proteins and proteins regulating the cell cycle, cell repair or the cytoskeleton (THORNBERRY and LAZEBNIK 1998). Interestingly, caspase inhibitors can abolish the completion of apoptosis in *Y. enterocolitica*-infected J774 cells, whereas exposure of phosphatidylserine at the cell surface, an early apoptosis marker, cannot be inhibited. Hence, YopP/YopJ might act upstream of caspases (Fig. 4).

Infection with wild-type *Yersinia* spp. has been shown to inhibit production of interleukin (IL)-8 and tumour necrosis factor (TNF) by different cell types (BEUSCHER et al. 1995; SCHULTE et al. 1996). TNF and IL-8 production requires activation of both NF-κB and MAP kinases and, consequently, YopP also seems to be responsible for the downregulation of TNF-α and IL-8 production caused by wild-type *Yersinia* spp. in intestinal cells and macrophages (BOLAND et al. 1998a; PALMER at al 1998).

An important question relates to the intracellular target of YopP, which presumably represents a molecule at the branch point of different signal-

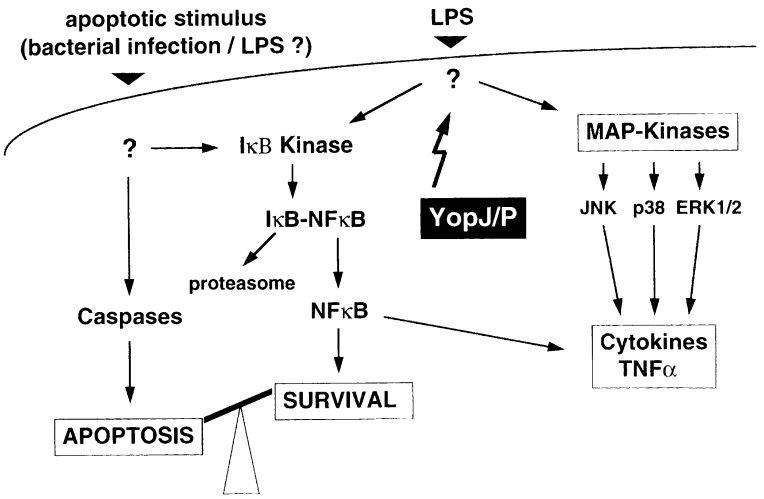

Fig. 4. Apoptosis signalling pathways affected by YopJ/P. *LPS* lipopolysaccharide, *NFκB* nuclear transcription factor κB, *IκB* inhibitory κB, *JNK* c-jun N-terminal kinase, *ERK* extracellular mitogen-regulated kinase

transduction pathways. Because of its drastic effects on cultured cells, the role of YopP/YopJ for virulence of *Yersinia* in animal models needs to be re-evaluated with a focus on macrophage-dependent immune responses.

IV. YopT, Another Actin-Disrupting Cytotoxin

YopT is a newly described 35.5-kDa protein whose presence may or may not be required for *Yersinia* virulence in the animal model (Iriatre and Cornelis 1998b). The only reported homology is to the C-terminal end of p76, an immunoglobulin-binding protein from *Haemophilus somnus* (Cole et al. 1993). Infection of HeLa cells with a *Y. enterocolitica* strain mutated in the five effector Yops (H, O, P, E and M) still caused disruption of the actin cytoskeleton, an effect which was abrogated when YopT was mutated in addition to the other Yops. Thus, YopT seems to be yet another effector Yop which, similar to YopE, disrupts the actin cytoskeleton. The cell-shape changes thought to be produced by YopH or the protein kinase YopO (YpkA) differ from the phenotypes produced by YopT or YopE (Rosquist et al. 1990; Hakansson et al. 1996; Iriarte and Cornelis 1998b). However, it has to be emphasised that, with the techniques used so far, it was not determined whether YopE needs YopT to produce a cytotoxic effect or whether YopE and YopT act on identical or separate targets of the same regulatory system. (see page 685)

V. YopM – So Far, no Evidence for an Intracellular Function

YopM is an acidic, 41-kDa protein (predicted isoelectric point = 4.09) that is required for virulence (Leung et al. 1990). YopM contains six repeated motifs and a number of leucine-rich repeats (LRRs) that are 19 amino acids long and related to the very common LRRs implicated in protein–protein interactions (Leung and Straley 1989; Kobe and Deisenhofer 1994). Probably due to a different number of LRR repeats, YopM exists in various isoforms having different mobilities in SDS-PAGE, which depend on the *Yersinia* species and serotype (Heesemann et al. 1986a; Boland et al. 1998b). Proteins homologous to YopM include the α-chain of glycoprotein Ib (a thrombin and von Willebrand-factor-binding protein) from platelets and IpaH, a Shigella protein of unknown function (Leung and Straley 1989). Through its common LRRs, YopM is weakly homologous to a variety of proteins with different functions located in different subcellular locations. YopM's similarity to glycoprotein Ib and initial observations of an extracellular location suggested that YopM might function by binding to extracellular thrombin. In fact, it was shown that released *Y. pestis* proteins containing YopM bound thrombin, but the released proteins from a YopM mutant did not (Leung et al. 1990). Furthermore, purified YopM could bind to thrombin in vitro and was able to inhibit thrombin-stimulated platelet aggregation (Reisner and Straley 1992). However, the assumed structural basis for the binding of YopM to thrombin became questionable when it was demonstrated that the thrombin-binding domain of

GPIb-α lies outside the region of homology to YopM (De Marco et al. 1994). So far, no thrombin-binding site in YopM has been characterised. In addition, by using YopM fusion proteins and digitonin-induced release of Yops from the cytosol of infected cells, it was proposed that YopM is mainly cytosolic (Boland et al. 1996; Lee et al. 1998). As is the case for Yop E, H, P and O, effective translocation of YopM was dependent on the "translocator" Yops B and D and on the "regulator", YopN (Boland et al. 1996). Although these data do not exclude an extracellular thrombin-binding activity of YopM, they are more consistent with the idea that YopM is an intracellular "effector" acting by an unknown mechanism.

VI. YpkA, a Putative Serine/Threonine Kinase Affecting Cell Shape

YpkA from *Y. pseudotuberculosis* is a 82-kDa protein essential for virulence in the mouse model (Galyov et al. 1993, 1994). The corresponding YopO from *Y. enterocolitica* is almost identical to YpkA on the amino acid level. The N-terminus of YopO/YpkA has considerable homology to eukaryotic serine/threonine protein kinases and to tyrosine kinases (Hanks and Quinn 1991; Galyov et al. 1993). Addition of [γ-^{32}P] adenosine triphosphate (ATP) to a concentrate of proteins released from *Y. pseudotuberculosis* caused labelling of YpkA. This is obviously due to autophosphorylation, because YpkA immunoprecipitated from Yersiniae-released proteins incorporated ^{32}P into serine in the presence of [γ-^{32}P]ATP. Released YpkA in which the last 73 amino acids were deleted or in which a fragment from the presumed catalytic domain (amino acids 207–388) was removed showed no autophosphorylation, indicating that the C-terminus and the putative catalytic domain in the N-terminus are both necessary for the autophosphorylating activity (Galyov et al. 1993). Interestingly, the mutants deficient in autophosphorylation lost virulence in the mouse model (Galyov et al. 1993). To unambiguously show autophosphorylation and characterise the enzymatic properties of YpkA/YopO in detail, studies with purified proteins are warranted. No effect of a single *Y. pseudotuberculosis* YpkA mutant on cell shape could be detected unless YpkA was overexpressed by a mutant strain not expressing Yops H, M, E and K (Hakansson et al. 1996b). This likely reflects the low expression and translocation levels of YpkA and the profound effects on the cytoskeleton of YopE and YopH produced by wild-type *Y. pseudotuberculosis*, which might mask the YpkA effect. The *Yersinia* mutant overexpressing YpkA caused a contractile, stellate phenotype in HeLa cells, characterised by pronounced retraction fibres, which seemed clearly different from the YopE effect. Immunofluorescence studies revealed that translocated YpkA located specifically to the plasma membrane of the HeLa cells. Hence, at the plasma membrane, YpkA might interact with and/or phosphorylate a signalling protein that controls the cytoskeleton. However, a new Yop (YopT) which also seems to be expressed by *Y. pseudotuberculosis* was recently found to affect the cytoskeleton (Iriarte and Cornelis 1998b). Therefore, the exact contribution of YpkA to cell-shape

changes produced by the YopE/H/M/K mutant is not clear. Certainly, the effect of the YopE/H/M/K mutant that does not express YpkA must be compared more carefully with the effect of the YopE/H/M/K mutant overexpressing YpkA, and these effects should be combined with the effects of additional YopT mutants, provided that YopT is indeed expressed and functioning in the *Y. pseudotuberculosis* strain used in these studies.

D. Perspectives

The last 5 years have seen enormous progress in the elucidation of the molecular functions of the *Yersinia* type-III translocation system and of individual Yops translocated into eukaryotic cells via this system. A simplified model of the current ideas about Yersiniae interactions with eukaryotic cells is depicted in Fig. 5. Although the exact structure of the secretion/translocation apparatus spanning the eukaryotic plasma membrane and the inner and outer bacterial membrane is still unclear, various Ysc proteins seem to represent either structural or regulatory components of the type-III translocation machinery (Cornelis and Wolf Watz 1997; Hueck 1998). Sequence homologies suggest that the structure of the *Yersinia* type-III system might resemble the recently published structure of the *Salmonella* type-III system on the supramolecular level (Kubori et al. 1998). Further structural studies are warranted to clarify

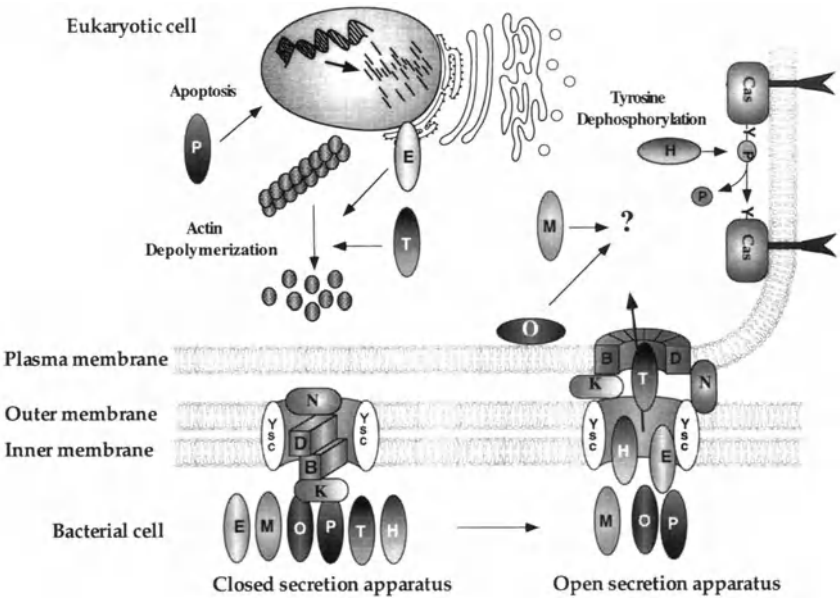

Fig. 5. Model of the Yop secretion/translocation machinery. *Ysc Yersinia* secretion, *B*, *D* translocator Yops, *K*, *N* regulator Yops; *H*, *T*, *P*, *O*, *M*, *E* effector Yops, *Cas* Crk-associated substrate

the organisation of the translocation pore at the protein level. In addition, molecules converting chemical energy into energy for protein translocation need to be characterised, and it should be analysed whether there is a requirement for protein folding and refolding during protein export and/or translocation, as is the case in eukaryotic protein import/export machinery. Major progress in this regard can be expected from cross-linking experiments identifying translocation intermediates, chrystallographical studies and reconstitution of the type-III system in vitro.

Equally important to the understanding of the type-III system on the molecular level is the question of exactly which eukaryotic regulatory mechanisms the different *Yersinia* effector Yops interfere with and, more specifically, which eukaryotic proteins these Yops bind to. The best investigated Yop in this regard is YopH, which specifically binds to a set of tyrosine-phosphorylated proteins (including paxillin and $p130^{Cas}$) via an N-terminal phosphotyrosine-binding module (Black et al. 1998). YopH is the only known Yop that has activity as a purified, recombinant protein. The major drawback of all the reports implicating YopE, YopP/YopJ, YopT and YpKA/YopO in specific cell functions is that the activities of individual Yops or plasmids expressing individual Yops were not investigated. These studies relied on recombinant *Yersinia* strains in which the Yop of interest was "knocked out" by transposon-mediated or -directed insertion of an antibiotic-resistance cassette or by in-frame deletions of large parts of single *yop* genes. More advanced *Yersinia* "injection systems" used strains in which all known effector Yops (H, O, P, E, M) except one (YopT) were mutated (Iriarte and Cornelis 1998b) or in which only single effector Yops were translocated (Roggenkamp et al. 1995). Even with these "designer strains", interpretation of single-Yop function is difficult to interpret due to potentially confounding effects brought about by *Yersinia* adhesin signalling through host cell receptors or by the translocation machinery itself, which can form pores in the eukaryotic plasma membrane and introduce translocators or regulators (like YopD and YopN) of unknown intracellular function.

Another question arises: namely, whether Yersiniae are able to compose a "menu" of individual Yops, which they translocate depending on which function they want to alter in any specific target cell. This would make sense in view of the ability of Yersiniae (1) to invade cells rather than remain extracellular (2) to reside in lymph follicles and lymph vessels rather than enter the bloodstream and (3) to eventually colonise one organ but not another. A molecular mechanism recently described adds to this possibility: it could be shown that a *lcrG* mutant was impaired in the secretion of all Yops, whereas a *tyeA* mutant was only impaired in the secretion of YopH and YopE (Iriarte et al. 1998a). LcrG and tyeA are proteins known to control Yop secretion, and the described phenomenon gives the first indication that translocation could be regulated Yop-specifically.

In conclusion, further investigation of the function of the *Yersinia* type-III secretion system and of the effector Yops will be useful and worthwhile for

several reasons. (1) An exact knowledge of the common strategies pathogenic bacteria use to establish infection, particularly bacteria that inhibit host immune functions, is a prerequisite to the development of new therapeutics. (2) Specifically engineered *Yersinia* strains could be used to deliver proteins of interest into eukaryotic cells (for example, to analyse the intracellular function of a protein or for vaccination purposes). (3) Yops might prove to be new, highly specific tools to study basic cellular mechanisms like vacuolar trafficking, cytoskeletal rearrangements or apoptosis. *Yersinia* modulins might, therefore, become similarly indispensable for cell-biological and clinical research like many of the bacterial toxins and modulins of different origins described in this book. (4) Finally, proteins of the type-III secretion system, which is widely distributed among pathogenic bacteria and, more specifically, Yop effector proteins, should be considered as targets for entirely novel therapeutic agents against infectious diseases.

While this manuscript was inpress we identified the GTPase RhoA as target of YopT. YopT covalently modifies and inactivates RhoA by an unknown mechanism (Zumbihl et al. 1999, J Biol Chem 274:29289–29293)

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