Reviews of

119 Physiology Biochemistry and Pharmacology

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With 18 Figures and 3 Tables

Springer-Verlag
Berlin Heidelberg New York London Paris
Tokyo Hong Kong Barcelona Budapest

ISBN 3-540-55192-1 Springer-Verlag Berlin Heidelberg New York
ISBN 0-387-55192-1 Springer-Verlag New York Berlin Heidelberg

Library of Congress-Catalog-Card Number 74-3674

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Typesetting: K+V Fotosatz GmbH, Beerfelden
Printing and binding: K. Triltsch GmbH, Würzburg
27/3130-5 4 3 2 1 0 – Printed on acid-free paper

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Rev. Physiol. Biochem. Pharmacol., Vol. 119
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A Retrospective on Transformation, Growth Control, and Some Peculiarities of Lipid Metabolism

H. DIRINGER[1] and R. FRIIS[2]

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1 Introduction

Transformed cells exhibit numerous aberrant properties, i.e., the elevated transport of nutrients, loss of contact inhibition of cell movement and cell division, reduced surface adhesiveness, increased membrane fluidity, loss of intercellular communication, and appearance of new tumor-specific antigens (Nicolau et al. 1978; Weber and Friis 1979; Boschek et al. 1981). Some of these properties result from molecular changes at the cell surface of transformed cells involving glycoproteins, glycolipids, and mucopolysaccharides as indicated by altered surface charges, sialic acid content, and agglutination characteristics (for a review see Hakomori 1973). In consequence, disturbances in growth regulation by and dependence on serum factors and hormones are observed.

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Even 12 years ago, turnover studies on cell membrane constituents were meager according to Black (1980) in his review on the shedding of proteins, glycoproteins, glycolipids, and glucosaminoglycans from the surface of normal and tumor cells. Yet the turnover of membrane constituents is of obvious importance in the regulation and control of cell growth. In fact, it is the turnover of various receptors with their sensory elements at the cell surface and regulatory domains inside the cell which now seems likely to be the most important regulatory system controlling normal cell growth.

In this communication we will restrict discussion to studies dealing with the turnover of lipids in normal and tumor cells using cell culture models. These studies have not only extended our understanding of the biosynthesis and function of phospholipids, but at the same time have demonstrated that the turnover of a particular phospholipid, phosphatidylinositol (PI), which has been of interest to biochemists and physiologists for decades (for review see Michell 1975) is in fact fundamental for the regulation of cell growth.

2 Phospholipids: Essential Function and Structure

The fundamental role of phospholipids in nature depends on their ability to form bilayers in a hydrophilic environment. Therefore, in evolution these molecules have become the essential constituents of cellular membranes. They represent the basic functional matrix for separating different cellular compartments from each other. This ability depends on the nonpolar interactions of the fatty acid chains, which are contained in these amphipathic molecules. The basic model of cellular membranes is that of a fluid mosaic of restricted and regulated permeability allowing the association of the lipids with proteins and glycoproteins, additional important constituents of membranes. The latter either span the lipid bilayer, are inserted into it, or are loosely associated with the lipids (Singer and Nicolson 1972). Besides some merely structural proteins, many enzymatically active proteins are present. Membrane-bound enzyme activities very often depend on an association with phospholipids. The association may be quite unspecific, i.e., any phospholipid will suffice, or very specific, i.e., the enzyme molecule is structurally associated with a very specific phospholipid moiety (Sandermann 1978). These two very important functions of phospholipids, their ability to form bilayers and to combine with proteins, we can define as structural functions.

The major structural differences among the phospholipids are to be found in the hydrophilic part of the molecule, where compounds like in-

Table 1. Major phospholipids of mammalian cells

Name	Abbreviation	Hydrophobic part	Hydrophilic part	Relative content[a]
Phosphatidylinositol	PI	Diacylglycerol	Phosphorylinositol	5–10
Phosphatidylserine	PS	Diacylglycerol	Phosphorylserine	5–15
Phosphatidylethanolamine	PE	Diacylglycerol	Phosphorylethanolamine	10–30
Phosphatidylcholine	PC	Diacylglycerol	Phosphorylcholine	30–60
Sphingomyelin	S	erythro-Ceramide	Phosphorylcholine	3–12

[a] These data represent approximations of the overall content of phospholipids extracted from various tissues. There are substantial differences with respect to the organ of origin and even more so to the different cellular membranes (Esko and Raetz 1983)

ositol, serine, ethanolamine, and choline are esterified with phosphoric acid (Table 1). Two phospholipids, phosphatidylcholine (PC) and sphingomyelin (S) contain the same polar group, phosphorylcholine, but differ in the nonpolar part of the molecule. This will be an important issue later in this discussion. The phosphoric acid in a phospholipid molecule is also esterified to the hydrophobic part of the molecule, which is most often diacylglycerol, but in the case of S, is erythroceramide. For the moment it is not especially important for us to remember that there is a microheterogeneity in the fatty acid composition of the diacylglycerol backbone in all phospholipid classes. Indeed, within PC and phosphatidylethanolamine (PE) some molecules may contain even an ether- or α,β-unsaturated ether unit instead of an acyl residue resulting in subfractions called ether lipids or plasmalogens, respectively. Also the special functions of arachidonic acid, a major fatty acid constituent of PI, are not of importance for purposes of this review.

3 Kinetics of Phospholipid Metabolism in Growing Cells: A Specific Function of PC

From the structure of phospholipids (Table 1), it is obvious that for studying the dynamics of the turnover of individual phospholipids, as well as their interrelations with each other, labeling with [^{32}P]orthophosphate would be appropriate. Alternatively, one can study the relative dynamics of turnover of the phosphate group as compared to that of inositol, serine, ethanolamine, or choline, or to the turnover of the fatty acid-containing

nonpolar moiety, by using a double-labeling technique. Pasternak and Bergeron (1970) labeled tissue culture cells with [^{3}H]choline or [^{3}H]inositol and reported that the turnover of phospholipids takes place in a biphasic pattern with breakdown of the entire lipid molecule. This turnover was thought to be independent of cell growth.

Much more detailed results were obtained later when phospholipids of growing tissue culture cells were labeled with [^{32}P]orthophosphate, the individual phospholipids separated, and the uptake of label during the pulse period, as well as during an extended chase period, was analyzed. The data obtained gave fundamentally new insights into the biosynthesis and function of phospholipid metabolism (Diringer 1973). Figure 1 shows such an experiment. Indeed, it was found that the total phospholipid turnover exhibited a biphasic pattern. It became obvious that labeling with [^{3}H]choline or [^{3}H]inositol as performed earlier (Pasternak and Bergeron 1970) must necessarily have given this result since only PC, the most abun-

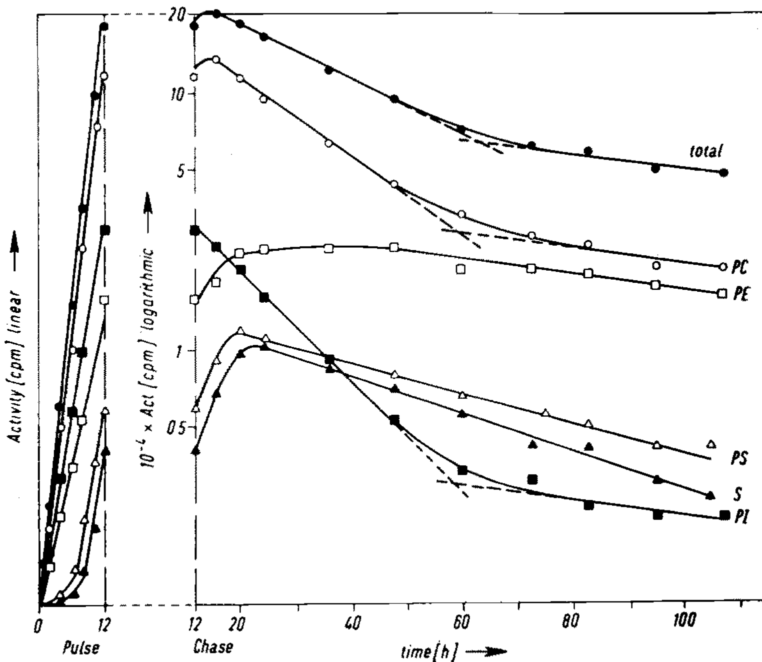


Fig. 1. The kinetics of [^{32}P]orthophosphate uptake and release from total and individual phospholipids in a pulse-chase experiment. Phospholipids were isolated from exponentially growing STU-51A/232B cells with a doubling time of 32 h. The ordinate shows radioactivity present in the cells in 10 ml of suspension culture. The data are corrected for the dilution introduced at 47 and 72 h. The radioactivity incorporated during the pulse period is plotted linearly with different scales for each lipid. The highest value in the linear plot is identical with that given in the logarithmic scale used for the chase period

dant choline-containing phospholipid, and PI turn over in just this fashion.

The kinetics observed in the experiment represented in Fig. 1 allows several very different phases to be distinguished:

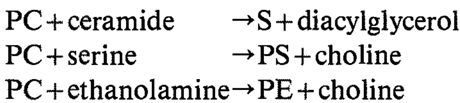
1. The pulse phase, during which three lipids, PC, PI, and PE incorporate the radioactive phosphate almost at once, whereas S and phosphatidylserine (PS) show a very delayed uptake.
2. The early/middle chase period (12–60 h after the start of the experiment), where three lipids, S, PS, and PE, continue to incorporate radioactive for at least 10 h, but where PC and PI lose radioactivity quite rapidly.
3. The very late chase period (past 60 h) were all the phospholipids turn over at a constant slow rate.

Let us analyze this experiment starting with the late chase phase, when all the phospholipids turn over slowly. We can assume that these labeled molecules are those that are related to the general function of all phospholipids, i.e., structural function. In making this assumption, it is implicit that we ascribe an extra function for those molecules in the PC and PI fractions which turn over rapidly. What could this extra function be?

The initial clue comes from a comparison of PC and S, i.e., the two phospholipids which contain the same polar group (phosphorylcholine), but have different hydrophobic backbones (diacylglycerol or erythroceramide). Both phospholipids have long been suspected to gain their polar head group from the same precursor molecule cytidine diphosphate choline (CDP-choline; Scribney and Kennedy 1958) the physiologic concentration of which is roughly about 1% of either S or PC (Diringer 1973). Although in vitro synthesis has established this pathway for PC (Kennedy and Weiss 1956), S synthesized in vitro was of the threo- rather than the natural erythro-configuration (Scribney and Kennedy 1958). The kinetics (Fig. 1) are in perfect agreement with the postulated pathway of PC (uptake of ^{32}P via a small precursor pool). The kinetics argue, however, against such pathway for the synthesis of S. The long lag-period of uptake of radioactivity into S during the pulse as well as the continuous uptake of [^{32}P]phosphate during the early chase period demand a different precursor pool for the biosynthesis of S, a precursor pool of considerable size as well. Actually there is only one such large pool of a possible precursor which could donate the phosphorylcholine group for the biosynthesis of S. This pool is the phospholipid PC. A more detailed analysis of the specific activities of PC and S (Diringer et al. 1972) had pointed to this pathway previously. Enzymatic studies (Diringer and Koch 1973; Ullman

and Radin 1974) ultimately proved the validity of this pathway for the biosynthesis of S, and this concept is now generally accepted (Esko and Raetz 1983; Kishimoto 1983).

As indicated by the kinetics of Fig. 1, PS is also synthesized indirectly (same delay of labeling and release or radioactivity during pulse and chase period, respectively, as with S). Exchange of choline in PC or inositol in PI against serine would explain such indirect kinetics of incorporation. Again, a comparison of the time course of the specific activities favors PC as the immediate precursor for PS im mammalian cells (Diringer 1973). Recently the biosynthesis of PS via PC in intact mammalian cells has been elegantly demonstrated using cell mutants (Kuge et al. 1986; Voelker and Frazier 1986). Thus, the extra function predicted for the PC molecules with rapid turnover is their involvement as precursors in the biosynthesis of other phospholipids, i.e., in the biosynthesis of S, PS, and to some extent of PE (Diringer 1973):


PC + ceramide → S + diacylglycerol
PC + serine → PS + choline
PC + ethanolamine → PE + choline

What then is the predicted extra function responsible for enhanced PI turnover?

4 Kinetics of Phospholipid Metabolism in Normal and Tumor Cells Under Unrestricted and Restricted Growth Conditions: A Specific Function of PI

Kinetic studies using double labeling techniques with [^{3}H]glycerol for the nonpolar backbone diacylglycerol and [^{32}P]phosphate for the adjacent polar head groups demonstrated that the extra function for PI must be quite peculiar (Diringer 1973). Figure 2 presents the ratios of ^{3}H (hydrophobic part) and ^{32}P (polar head group) measured for the various phospholipids during the pulse and chase periods.

A comparison of this ratio during the chase period for PC, PI, and PS again demonstrates that PC, rather than PI, must be the precursor in the biosynthesis of PS. Much more interesting, however, was the observation that only in PI, but not in any other phospholipid, was the ^{3}H$-$^{32}P ratio increased during the chase period in growing cells. This result tells us that the polar head group of some fraction of the PI molecules turns over more rapidly than the nonpolar diacylglycerol backbones. The explanation cannot be the recycling through a diacylglycerol pool common for all phos-

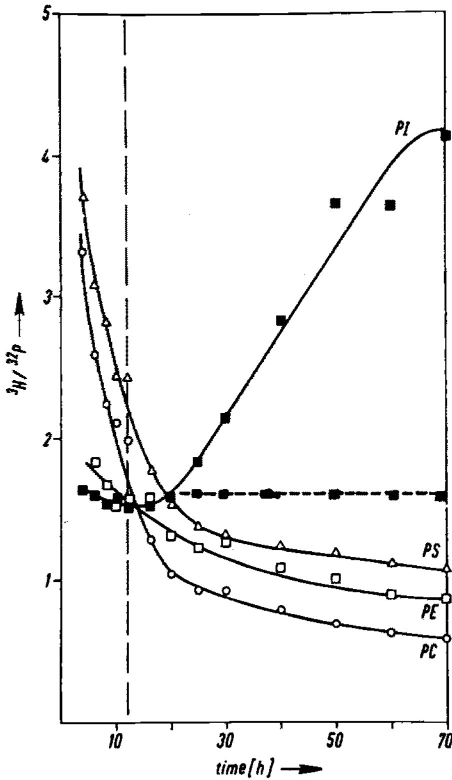


Fig. 2. The $^3H-^{32}P$ ratio of individual phospholipids is shown in a parallel experiment, like that shown in Fig. 1, but employing [^{3}H]glycerol in combination with the [^{32}P]orthophosphate. The *dashed line* indicates the ratio for PI under conditions of restricted growth owing to high cell population density or serum deprivation

pholipids since the other phospholipids show very different ratios of label. Thus, a compartmentalization of PI molecules involved in this process has to be postulated. In other words, the PI molecules showing rapid turnover must have been incorporated into very specific areas of cellular membranes where reutilization of diacylglycerol can be restricted to the biosynthesis of PI only. This compartmentalization of PI suggests a very specific function for this phopholipid (Diringer 1973).

Between the discovery that carbamylcholine or acetylcholine treatment of pancreas slices stimulated incorporation of [^{32}P]phosphate into phospholipids (Hokin and Hokin 1953) and the review of Michell (1975) an extensive collection of data on phospholipid turnover had accumulated. Although in an article on biochemical aspects of the function of the action of acetylcholine on nerve cells it had already been assumed that the diglyceride moiety is stable and is bound to the membrane during PI degradation, the main consequence of PI turnover was thought to be enhanced membrane permeability (Durell et al. 1969). Only when Lapetina and Michell (1973) and subsequently Michell (1975) drew attention to the

fact that there was some evidence that this peculiar turnover of PI might be associated with the plasma membrane, and when in the latter publication, the hypothesis was enunciated that PI turnover might generally be associated with signal transduction via cell surface receptors resulting in the release of a water-soluble second messenger from the PI molecule, (which in turn would trigger Ca^{2+} in release from storage compartments and consequently activate Ca^{2+}-dependent enzyme reactions), only then had a unifying concept for the role of PI turnover emerged.

In those days this hypothesis was provocative and many biochemists remained critical. Michell, however, continued to pursue and extend his hypothesis to include regulation of cell division and differentiation events (Michell 1982) basing his argumentation mainly on PI turnover data obtained following stimulation of lymphocytes (Fisher and Mueller 1968), and in growth studies of normal cells and tumor cells (Koch and Diringer 1973a; Diringer and Friis 1977).

Fisher and Mueller (1968) had described an imediate increase of incorporation of [^{32}P]phosphate into PI following stimulation of lymphocytes with phytohemagglutinin. This increased uptake of label into the lipid was thought to be due to stimulated synthesis of PI rather than breakdown. As suggested later (Alan and Michell 1977), elevated breakdown better explains the phenomenon.

A clear correlation between cell growth and PI turnover was demonstrated in kinetic studies with normal (Koch and Diringer 1973b) and DNA tumor virus (SV40)-transformed (Koch and Diringer 1973c) mouse fibroblasts, on the one hand, and with normal and Rous sarcoma virus (RSV)-transformed quail cells (Diringer and Friis 1977) on the other. In particular, the latter system has the advantage that the *src*-oncogene-induced transformation can be induced within 2–3 days and normal quail cells show a striking growth inhibition at high cell density or in serum-deficient media.

When the composition and metabolism of phospholipids in such cells, previously labeled with [^{32}P]phosphate and [^{3}H]glycerol, was investigated during the chase period under conditions of restricted versus exponential growth, the following results emerged:

1. Regardless of transformation or growth conditions, no statistically significant change in phospholipid composition was detected.
2. No difference in the labeling of S or PS was observed in connection with either growth or transformation.
3. A decreased breakdown of PC and a decreased uptake of radioactivity into PE was observed at high cell density or under conditions of serum depletion, but was not related either to cell growth or transformation.

4. Turnover of PI was the only parameter that strictly correlated with cell growth in normal as well as in the virally transformed cells.

This growth-related change in PI turnover was shown to be due to an enhanced turnover of the polar head group of the PI molecule. In Fig. 2 this is indicated by the dotted line, which refers to the unchanged $^{3}H - ^{32}P$ ratio seen in PI with normal cells, but not with tumor cells, subjected to conditions of high cell density or serum deprivation. Consequently, transformation of a normal cell by a tumor virus results in the loss of the density-dependent or serum-dependent regulatory mechanisms which restrict the growth of normal cells. The extra function of PI turnover is therefore related to growth regulation and the release of a second messenger from PJ is likely to occur.

5 Perspectives

The first water-soluble second messenger suspected of stimulating Ca^{2+} release (Lapetina and Michell 1973) or initiating DNA replication (Koch and Diringer 1974) was *myo*-inositol-1,2-cyclic phosphate. Experimental evidence for this hypothesis has, however, never been obtained (Diringer et al. 1977). It has now emerged from the initial experiments of Berridge and Irvine (1984) that the water-soluble second messenger enhancing the release of Ca^{2+} is inositol-1,4,5-trisphosphate. Equally important as this discovery was the observation of Nishizuka (1984) of another second messenger derived from PI turnover, i.e., the diacylglycerol residing in the plasma membrane. This compound stimulates protein kinase C to perform its function at low Ca^{2+} levels. With this discovery PI turnover has become linked to hormonal control of cell growth and to the action of oncogenes involved in the disturbance of cell growth regulation (Kikkawa and Nishizuka 1986). Thus, pathways for lipid metabolism take on relevance in the framework of cell regulatory processes controlling both proliferation and differentiation.

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Rev. Physiol. Biochem. Pharmacol., Vol. 119
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Phospholipases C and D in Mitogenic Signal Transduction

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1 Introduction

The receptor-mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P_2] is now recognised as a widespread signal transduction pathway, the major elements of which are conserved throughout eukaryotes from yeast to man. This pathway is utilised by a variety of hormones and neurotransmitters (reviewed by Downes and Michell 1985; Berridge 1987a; Berridge and Irvine 1989) as well as mitogenic growth factors (Berridge 1987b; Whitman and Cantley 1988). The two products of phosphoinositidase C-(PIC)-catalysed PtdIns(4,5)P_2 hydrolysis are both second messengers; inositol-1,4,5-trisphosphate [Ins(1,4,5)P_3] binds to a specific intracellular receptor and releases sequestered calcium from a subpopulation of the endoplasmic reticulum while *sn*-1,2-diradylglycerol (DG) forms a complex with phosphatidylserine (PtdSer) and Ca^{2+} and activates protein kinase C (PKC). The physiological role of both molecules has been reviewed extensively (Berridge and Irvine 1989; Kikkawa et al. 1989; Parker et al. 1989; Villereal, this Vol.). Recently, it has become apparent that other phospholipid classes, notably phosphatidylcholine (PtdCho), are hydrolysed in an agonist-dependent manner and may give rise to DG and other biologically active lipids (reviewed by Exton 1990, Billah and Anthes 1990). In this article we discuss recent insights into the role of inositol lipids in mitogenic signal transduction, the mechanisms by which activated receptors couple to stimulated inositol lipid metabolism and review the evidence for multiple sources of DG in mitogenic signalling systems. We also consider the role of DG and other lipids derived from PtdCho hydrolysis, notably phosphatidic acid (PtdOH) and arachidonic acid, as potential second messengers. For convenience we will consider growth factors in two broad categories: those which couple to effector systems via a classical receptor-G-protein-effector amplification cascade (RGE-type; Pouysségur 1990) and those which couple to effector systems via a ligand-activated receptor tyrosine kinase activity (RTK-type; Ullrich and Schlessinger 1990). This classification reflects the model systems on which the majority of the literature is based. We will, however, also consider the possible role of proto-oncogene products in regulating phospholipase function and other non-mitogenic systems as comparisons. In addition we discuss the role of lipid-signalling pathways within the framework of multiple pathways for mitogenic signal transduction (Rozengurt 1985, 1986; Whitman and Cantley 1988).

2 Inositol Lipids and Mitogenic Signal Transduction

The observation that a variety of growth factors stimulate the hydrolysis of PtdIns(4,5)P_2 and generation of Ins(1,4,5)P_3 and DG implicates a known signal transduction pathway in the mediation of proliferative responses to mitogens as diverse as bombesin, platelet-derived growth factor (PDGF) and foreign antigen acting at the T-cell receptor. Furthermore, this signal transduction pathway mediates many of the early events in the prereplicative phase of the mitogenic response such as transient increases in $[Ca^{2+}]_i$ (Berridge and Irvine 1989; Villereal, this issue), Na^+/H^+ exchange (Pouysségur this issue), induction of the competence genes c-*fos*, c-*myc* and c-*jun* and activation of the ribosomal protein S6 kinase (Thomas, this issue). In addition, there is evidence that microtubule-associated protein (MAP) kinase and Raf-1 kinase, two serine/threonine protein kinases which act as integration points for different signalling pathways, are also targets for activation by PKC. Evidence for a major role for PKC in mitogenesis is underlined by the recognition of PKC as the cellular receptor for the DG mimetic, tumour-promoting phorbol esters (Castagna et al. 1982). In addition, over-expression of PKC in fibroblasts causes morphological transformation, enhanced growth rates (especially at limiting serum concentrations), increased saturation growth densities and increased tumorigenicity when injected into nude mice (Persons et al. 1988; Housey et al. 1988). Microinjection of DG stimulates BALB/c 3T3 cells to re-enter the cell cycle and is co-mitogenic with insulin (Suzuki-Sekimori et al. 1989), whilst antibodies to PtdIns(4,5)P_2 abolish PDGF and bombesin-stimulated mitogenesis (Matuoka et al. 1988). Kinase-defective mutants of PDGF and colony-stimulating factor 1 (CSF-1) receptors which have impaired mitogenic signalling capacity also have impaired association with inositol lipid 3′-kinase activity.

In contrast, some observations argue against the stimulated hydrolysis of inositol lipids being *necessary* or *sufficient*, on its own, to mediate a full mitogenic response (see Whitman and Cantley 1988). The simplest of these is that stimulated inositol lipid hydrolysis is not *necessary* for many growth factors to exert their mitogenic effect, e.g. CSF-1 (Imamura et al. 1990). For RGE-type growth factors such as bombesin, the generation of Ins(1,4,5)P_3 is essentially transient (Cook et al. 1990) and this correlates with the transient increase in $[Ca^{2+}]_i$ (Lopez-Rivas et al. 1987). Despite this, many of the events associated with transit through G_1 of the cell cycle are more sustained, e.g. increased pH_i (Bierman et al. 1990), phosphorylation of the myristylated, alanine-rich, protein C kinase substrate (MARCKS) protein (Takuwa et al. 1991) and occur a considerable time after the addition of growth factor. Perhaps the most convincing study has

been that of Seuwen et al. (1990) in the hamster fibroblast cell line (CCL39). In these cells α-thrombin is a potent mitogen and is able to activate inositol lipid hydrolysis, inhibit adenylyl cyclase and activate tyrosine phosphorylation of cellular proteins (Pouysségur 1990). To assess the role of inositol lipid hydrolysis in growth factor action the M_1 muscarinic acetylcholine receptor was transfected into CCL39 cells since this receptor couples to PIC without activating parallel signalling pathways. By selecting a clone in which carbachol stimulated inositol lipid hydrolysis to a similar extent as α-thrombin they showed that both agonists were able to stimulate the early biochemical events (increases in $[Ca^{2+}]_i$, pH_i, c-*fos* and c-*jun* expression) to the same extent, but only α-thrombin was mitogenic. They concluded that strong and persistent inositol lipid hydrolysis may elicit many of the early mitogenic signals but is not *sufficient* to stimulate or maintain cell proliferation (Seuwen et al. 1990). Thus, it seems that mitogen-stimulated inositol lipid hydrolysis may be of most importance in attaining a state of "competence" and will be mitogenic in cooperation with growth factors which stimulate other parallel pathways. The concept of multiple and redundant pathways of mitogenic signal transduction has been extensively reviewed (Rozengurt 1985, 1986; Whitman and Cantley 1988). Figure 1 summarises the activation of mitogenic pathways by e.g. bombesin in Swiss 3T3 cells.

3 The Coupling of Activated Receptors to Stimulated Inositol Lipid Metabolism

Despite extensive research there is still no definitive second messenger role attributed to any inositol-containing compound, other than $Ins(1,4,5)P_3$ (reviewed by Berridge and Irvine 1989). The suggestion that inositol-1,3,4,5-tetrakisphosphate [$Ins(1,3,4,5)P_4$] acts to regulate Ca^{2+} entry across the plasma membrane (Irvine 1990) remains contentious though $Ins(1,3,4,5)P_4$ has recently been shown to increase $[Ca^{2+}]_i$ in SH-SY5Y neuroblastoma cells (Gawler et al. 1990); certainly mass analysis reveals that $Ins(1,3,4,5)P_4$ levels do increase considerably upon agonist stimulation. The levels of an inositol tetrakisphosphate ($InsP_4$), apparently $Ins(3,4,5,6)P_4$, are elevated in v-*src*-transformed cells but the significance of this phenomenon remains to be defined. Much attention has focussed recently upon the role of 3′-phosphorylated inositol lipids particularly since an inositol lipid 3′-kinase activity has been shown to associate with activated growth factor receptors in a ligand-dependent manner (see below). However, there is, as yet, no evidence for any biological function

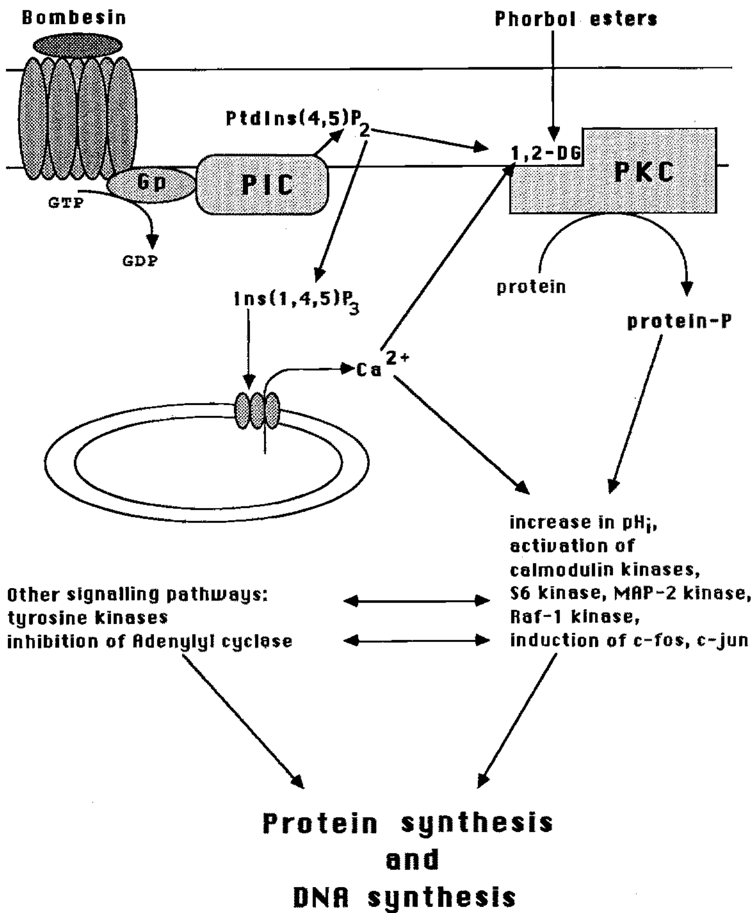

Fig. 1. Mitogenic stimulation of PtdIns(4,5)P_2 hydrolysis. Using bombesin in Swiss 3T3 cells as an example the pathways that may lead to the activation of mitogenesis are outlined

for these lipids. In the last 2 years there have, however, been major advances in understanding how ligand-activated receptors are able to couple to the stimulation of inositol lipid metabolism.

3.1 Multiple Isoforms of PIC

In refering to the phospholipase C (PLC; EC 3.1.4.3) which hydrolyses PtdIns(4,5)P_2 we use the general term "phosphoinositidase C" (PIC), suggested by Downes and Michell (1985), unless dealing with a specific isoform, in which case we use the nomenclature of Rhee et al. (1989) and

Meldrum et al. (1991). The family of PICs has been reviewed extensively (Rhee et al. 1989; Meldrum et al. 1991) in much greater depth than can be afforded by the nature of this article.

The PICs are broadly divided into four classes α, β, γ and δ; this assignment was originally due to the naming of PLCs β, γ, and δ as the 154-kDa, 145-kDa and 85-kDa immunologically distinct proteins purified from bovine brain and subsequently cloned (Rhee et al. 1989). The overall identity between PLCβ_1, PLCγ_1 and PLCδ_1 is quite low but they possess two regions of high primary sequence homology, regions I and II, which are likely to contribute to the catalytic domain of the enzymes. Disruption of either region, by deletions or selected point mutations, results in a complete loss of catalytic activity (see Meldrum et al. 1991).

The high degree of homology within the I and II regions has allowed the cloning of novel PIC cDNAs by low-stringency hybridization and sequence analysis has confirmed the validity of the original assignment of β, γ and δ classes. Analysis of novel cDNA clones and immunological comparisons has allowed assignment to a particular class and within these classes the enzymes exhibit much greater homology to each other. Thus, to date there appear to be two forms of PLCβ (β_1 and β_2), two forms of PLCγ (γ_1 and γ_2) and three forms of PLCδ (δ_{1-3}). PLCα does not possess sequence homology with any of the PIC forms previously isolated from bovine brain and the cDNA does not encode a catalytically active protein. In general, polyphosphoinositides are the preferred substrates for the PLCβ_1 and δ_1 at low or physiological [Ca^{2+}] although δ_1 and δ_2 can hydrolyse phosphatidylinositol (PtdIns) at millimolar concentrations of Ca^{2+}. In contrast, PLCγ_1 appears to exhibit similar activity against PtdIns and PtdIns(4,5)P_2 at physiological [Ca^{2+}].

Meldrum et al. (1991) speculate that regions outside the conserved sequences I and II may be candidates for specific interactions with other regulatory components in signal transduction, i.e. the guanine nucleotide regulatory protein, G_p, activated agonist receptors or inhibitory components such as a putative inhibitory G-protein (G_pi). There is strong evidence that this is indeed the case, particularly for PLCγ_1 in which the I and II regions are divided by a 500-amino-acid stretch which possesses two SH2 regions implicated in association with phosphorylated tyrosine residues on the c-*src* protein and various growth factor receptors (see below). Indeed PLCγ_1 is unique among the PICs in associating with and being a substrate for growth factor receptor tyrosine kinases. Two recent reports have shown that G_q or a related protein is able to activate PIC at physiological [Ca^{2+}] (Smrcka et al. 1991; Taylor et al. 1991). Smrcka et al. (1991) have shown that G_q increases the affinity of a partially purified PLC preparation to the submicromolar range as well as increasing its

catalytic activity. Taylor et al. (1991) have shown that a purified G-protein α-subunit, GPA-42, which is immunologically related to G_q is able to activate PLCβ_1 but not γ_1 or δ_1; this is without effect upon the affinity of the enzyme activity for Ca^{2+}. Thus, a picture is emerging of distinct classes of PIC with defined catalytic activities and mechanisms of activation and regulation; consequently it seems likely that the diversity of PIC isoforms is an evolutionary adaptation to multiple pathways for receptor regulation of PIC activity.

3.2 The Identification of G_p, the Guanine Nucleotide Regulatory Protein Coupling Receptors to PIC

A variety of hormone and neurotransmitter receptors couple to activation of PIC in a guanine nucleotide-dependent manner (Cockcroft 1987). In much the same way many RGE-type growth factors couple to the activation of inositol lipid hydrolysis in a guanine nucleotide-dependent manner in permeabilised cell systems, e.g. bombesin-stimulated inositol phosphate accumulation in Swiss 3T3 cells (Plevin et al. 1990). Consistent with this the receptors for these growth factors possess the seven transmembrane spanning domains typical of receptors which couple to G-proteins, e.g. the bombesin receptor, which has recently been cloned from Swiss 3T3 cells (Battey et al. 1991). Despite this, the molecular identity of G_p, the G-protein-linking receptor to PIC, has remained elusive, not least because in some systems agonist-stimulated inositol lipid hydrolysis is pertussis toxin (P-tox) sensitive suggesting the involvement of a member of the G_i/G_o family. In many systems mitogenesis, stimulated by an RGE-type growth factor, is sensitive to P-tox but this does not correlate well with P-tox-sensitive inositol lipid hydrolysis (Taylor et al. 1988; Pouysségur 1990) suggesting that in these systems G_p is not a substrate for P-tox. Recently, candidates for this protein have been isolated by both classical protein purification and molecular biological methods. One of these proteins, purified by affinity chromatography with immobilised $\beta\gamma$-subunits (Pang and Sternweis 1990), is identical to the novel G-protein α-subunit, α_q, and similar to α_{11}, both isolated by PCR cloning using a mouse brain cDNA library (Strathmann and Simon 1990). Smrcka et al. (1991) have now reconstituted bovine brain PIC activity with G_q and shown that it both enhances the maximal activity of the enzyme and lowers the affinity of PIC for Ca^{2+} to approximately 0.1 μM, i.e. the approximate resting $[Ca^{2+}]_i$. Both α_q and α_{11}, and probably α_{14}, appear to constitute a new class of α-subunits for heterotrimeric G-proteins (Strathmann and Simon 1990); they exhibit poor sequence homology to the other α-subunits (α_s,

α_i and α_o), except in the conserved regions required for GTPase function, and are ubiquitously expressed. Thus it may well be that a new class of G-protein α-subunits, G_q, may represent the putative G_p responsible for regulating PIC; the diversity of members already established for this class may reflect a specificity for distinct PIC isoforms. Taylor et al. (1991) have recently shown that GPA-42 is able to specifically activate PLCβ_1. Since cDNA clones of both of these molecules are now available, future studies should allow site-directed mutagenesis and specific deletions and insertions in both proteins to assess the sites of interaction between G_p and PLCβ_1 and the nature of the specificity of coupling between G-proteins and effectors.

Both α_q and α_{11} possess the conserved arginine residue analogous to Arg-201 in Gα_s and the sequence Asp-Val-Gly-Gly-Gln-Arg including the Gln corresponding to Gln-227 in Gα_s; these residues are vital for GTP hydrolysis by α_s, and point mutations at this site abolish GTPase activity, resulting in elevated levels of cAMP and enhanced mitogenic responsiveness of a clone of Swiss 3T3 cells (Zachary et al. 1990). Mutations at these sites in α_s and α_i (the putative *gsp* and *gip*2 oncogenes) are found in human endocrine tumours such as the growth hormone-secreting pituitary tumours which exhibit elevated cAMP levels (the *gsp* mutation; Landis et al. 1989) and in some adrenal and ovarian tumours (the *gip*2 mutation; Lyons et al. 1990). The restricted tissue distribution of these mutations suggests that they do not play a general role in transducing mitogenic signals. In fact most cell types do not proliferate in response to elevations in cAMP level; the pituitary represents a cell type which does respond mitogenically to cAMP and the discovery of *gsp* mutations might therefore be considered somewhat fortuitous. Since G_p regulates a signal transduction pathway which is more widely implicated in the regulation of proliferation it will be of interest to see if point mutations in members of the α_q class, in regions analogous to the sites in *gsp*, are able to transform 3T3 cells or are found in certain tumours. Many of the RGE-type growth factors are neuropeptides (e.g. bombesin, vasopressin, endothelin); in the light of the marked tissue selectivity of *gsp* mutations potentially oncogenic $G_{p/q}$ mutations may be more prevalent in neuronal or neuroendocrine tumours.

3.3 Phosphorylation and Activation of PLCγ_1 by RTK

The mechanism by which growth factors of the RTK family, e.g. epidermal growth factor (EGF) and PDGF, stimulate PtdIns(4,5)P_2 hydrolysis is fundamentally different from that utilised by bombesin since it is not modulated by guanine nucleotide analogues (Catteneo and Vicentini 1989). In

the case of PDGF the generation of $Ins(1,4,5)P_3$ is much smaller than that for bombesin but is of longer duration (Plevin et al. 1991 a); this may relate to the inability of phorbol esters to inhibit PDGF-stimulated inositol phosphate accumulation. This is in marked contrast to the inhibition of bombesin-stimulated inositol phosphate production by phorbol myristate acetate (PMA) which may represent a PKC-mediated feedback inhibition pathway (Brown et al. 1987; Cook and Wakelam 1991).

Upon ligand-induced activation of the RTK domain both EGF and PDGF receptors physically recruit and phosphorylate a distinct isoform of PIC, PLCγ_1; this is associated with translocation of PLCγ_1 from the soluble to membrane fractions (see Meldrum et al. 1991). Recently it has been confirmed that tyrosine phosphorylation of PLCγ_1 increases its catalytic activity (Nishibe et al. 1990). In addition, a report from Goldschmidt-Clermont et al. (1990) has described a reconstitution of PLCγ_1 activity which requires not only EGF, EGF receptor (EGFR), $PtdIns(4,5)P_2$ and soluble enzyme but also the soluble actin-binding protein named profilin. Profilin binds up to five molecules of $PtdIns(4,5)P_2$, but not PtdCho, PtdSer or phosphatidyl ethanolamine (PtdEtn), with reasonably high affinity; this binding inhibits both the interaction between profilin and actin and the hydrolysis of $PtdIns(4,5)P_2$ by soluble PIC. Goldschmidt-Clermont et al. (1991) have now shown that in the presence of profilin, PLCγ_1 activity is dependent upon phosphorylation state; i.e. phosphorylation of PLCγ_1 by the EGFR relieves the tonic inhibition of PLCγ_1 activity by profilin. It is not clear how the phosphorylation of PLCγ_1 overcomes the inhibition by profilin, but once $PtdIns(4,5)P_2$ hydrolysis is initiated the released profilin may play a role in modulating growth factor-stimulated cytoskeletal rearrangements and membrane ruffling by virtue of its ability to bind to actin and prevent actin polymerization (Lassing and Lindberg 1985). Whether such a role applies to the activation of PLCγ_1 by the PDGF receptor (PDGFR) remains to be seen.

The physiological relevance of EGF-stimulated inositol lipid hydrolysis is subject to debate; in a variety of quiescent fibroblast cell lines EGF is a co-mitogen but is unable to stimulate inositol phosphate accumulation (Hesketh et al. 1988; Wright et al. 1990) suggesting that stimulated inositol lipid hydrolysis is not *necessary* for EGF-stimulated mitogenesis. EGF does stimulate InsP accumulation in systems where EGF receptors are over-expressed or are expressed in nonphysiological surroundings, e.g. the A431 epidermoid carcinoma cell line or the murine NIH3T3 cell line expressing human EGFR (HER14 cell line; Margolis et al. 1989). In the A431 cell EGF is able to stimulate InsP accumulation but actually inhibits growth (Iwashita et al. 1990). Levitski (1990) speculates that the coupling of EGFR to PLCγ_1 may be of most importance in cells over-expressing receptors or

possessing mutant receptors where the signal may contribute to the unrestrained growth exhibited by such cells. The coupling of PDGF receptors to PLCγ_1 appears to be a more relevant event in normal untransformed cells expressing their native receptor complement.

A major question which remains is how PLCγ_1 is recruited to the plasma membrane and associates with activated EGFR and PDGFR? The association with receptors appears to be mediated via interactions between SH2 (*src* homology) domains in PLCγ_1 and phosphorylated tyrosine residues on the receptor. SH2 regions appear to specifically recognize tyrosine-phosphorylated regions of proteins, e.g. the SH2 domain of p47$^{gag\text{-}crk}$ oncogene will not associate with pp60$^{v\text{-}src}$ if autophosphorylation at Tyr-416 is prevented. Thus, PLCγ_1 may be able to associate with PDGFR and EGFR following their ligand-activated autophosphorylation and this association may allow PLCγ_1 to be phosphorylated by the receptor-catalytic domain (see Cantley et al. 1991).

3.4 Activation of Inositol Lipid Kinase(s) and the Role of 3′-Phosphorylated Inositol Lipids

A number of agonists stimulate the rapid formation of two highly polar inositol lipids which are not part of the well-characterized canonical pathway (i.e. they do not possess the head group structure of PtdIns(4,5)P_2). Whilst the structure of three polyphosphoinositides has previously been established [PtdIns(4)P, PtdIns(3)P and PtdIns(4,5)P_2; Auger et al. 1989; Stephens et al. 1989] a number of studies suggest that the novel lipids are actually PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. However, until recently, the precise enzymatic pathway for the formation of these lipids remained obscure.

The association of an inositol lipid kinase activity with ligand-activated PDGFR and in immunoprecipitates of middle T antigen (MTAg) transformed cells was reported by Kaplan et al. (1987). The PtdIns kinase activity correlated with the presence of an 85-kDa protein which was also a substrate for the PDGF RTK. Whitman et al. (1988) showed that in MTAg-transformed cells a PtdIns was formed which was chromatographically identical to PtdIns(3)P. In PDGF-stimulated smooth muscle cells the time-course of production of two lipids, apparently PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, was consistent with the recruitment of a putative PtdIns-3′-kinase activity to the activated PDGFR. PtdIns-3′-kinase has recently been purified from rat liver (Carpenter et al. 1990). It consists of an 85-kDa subunit, the same protein as that found with activated PDGFR and MTAg, and a 110-kDa protein which form a native complex of 190-kDa.

Both subunits are required for activity and since the 85-kDa protein is found associated with PDGFR and MTAg it may exert a regulatory effect upon the holoenzyme. Thus it has been proposed that the novel 3′-phosphorylated inositol lipids may arise from the stimulated activity of PtdIns-3′-kinase acting upon PtdIns, PtdIns(4)P and PtdIns(4,5)P_2 (Whitman and Cantley 1988; Cantley et al. 1991).

There are a number of problems with many of these studies. First, the formation of PtdIns(3)P in vivo in cells chronically transformed by MMTAg does not mean that PtdIns(3)P is the initial product of the MTAg-associated kinase. Second, the structures of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 were, until recently, only putative assignments. Third, whilst the purified PtdIns-3′-kinase could phosphorylate PtdIns, PtdIns(4)P and PtdIns(4,5)P_2 in vitro it was by no means clear which was the preferred substrate in vivo. Finally, in those studies of the formation of putative PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 in agonist-stimulated intact cells, the time-course analysed was not sufficiently thorough to define which lipid was formed first.

These issues have been addressed in the recent study by Stephens et al. (1991) in f-Met-Leu-Phe-stimulated human neutrophils. Using a series of enzymatic and chemical assays they confirmed the structures of the two novel inositol lipids as indeed being PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. By analysing the relative rates at which [^{32}P] orthophosphate (Pi) was incorporated into each of the individual phosphate groups of the polyphosphoinositides they have shown that PtdIns(3,4,5)P_3 is actually formed by the 3′-phosphorylation of PtdIns(4,5)P_2. The [^{32}P]-labelling patterns of PtdIns(3)P and PtdIns(3,4)P_2 were ambiguous in that they could be obtained by the phosphorylation of PtdIns and PtdIns(4)P or by the dephosphorylation of higher phosphorylated inositol lipids. However, in intact neutrophils the production of PtdIns(3)P and PtdIns(3,4)P_2 lagged behind that of PtdIns(3,4,5)P_3 which was significantly above control 2–3 s after addition of f-Met-Leu-Phe. Finally, in neutrophil lysates PtdIns(3,4,5)P_3-5′-phosphatase and PtdIns(3,4)P_2-4′-phosphatase activities were readily detected. Thus, in neutrophils at least, the putative PtdIns-3′-kinase is in fact a PtdIns(4,5)P_2-3′-hydroxykinase. Preliminary estimates suggest that levels of PtdIns(3,4,5)P_3 may increase by as much as 40-fold in f-Met-Leu-Phe-stimulated neutrophils. Since PtdIns(3,4,5)P_3 formation is apparent within 2 s of f-Met-Leu-Phe addition it would appear that activation of PtdIns(4,5)P_2-3′-hydroxykinase is a rapid, receptor-driven event responsible for producing large, localised amounts of PtdIns(3,4,5)P_3. This may well constitute a novel signal transduction pathway (Stephens et al. 1991) and serves to underline the potential functional diversity of inositol-containing compounds afforded by their acknowledged structural diversity.

Unfortunately there is relatively little evidence to date indicating a precise function for PtdIns(3,4,5)P_3, or indeed any of the 3′-phosphorylated inositol lipids. None of the 3′-phosphorylated inositol lipids are substrates for PIC (Lips et al. 1989; Serunian et al. 1989) so that they are unlikely to serve directly as sources of DG or, in the case of PtdIns(3,4,5)P_3, Ins(1,3,4,5)P_4. Cantley et al. (1991) speculate that they may be substrates for phospholipase D (PLD; EC 3.1.4.4); however, it seems that PtdCho and not the inositol lipids are by far the preferred substrates for PLD (see Billah and Anthes 1990). In light of the effects of PtdIns(4,5)P_2 upon actin-profilin interactions (see above) it is possible that PtdIns(3,4,5)P_3 may exert effects upon actin filaments and cytoskeleton modifications (discussed by Cantley et al. 1991). Whilst it is well known that various actin-binding proteins associate with PtdIns(4,5)P_2 (e.g. profilin, myosin type I and glycophorin-band 4.1; see Cantley et al. 1991) the mass of PtdIns(4,5)P_2 changes by two- to three-fold in response to extracellular stimuli. The considerable increases in a highly charged lipid such as PtdIns(3,4,5)P_3 (Stephens et al. 1991) are likely to exert profound effects upon the inner leaflet of the plasma membrane and might be expected for this reason alone to modify cytoskeleton-membrane interactions; whether PtdIns(3,4,5)P_3 actually binds specifically to individual cytoskeletal elements remains to be seen, but if so this may contribute to the membrane ruffling events seen in response to many growth factors. Since PtdIns(3,4,5)P_3 is far less abundant than PtdIns(4,5)P_2 any specific binding to putative cellular receptors will be required to be of high affinity (Cantley et al. 1991).

In the context of mitogenesis the activation of PtdIns(4,5)P_2-3′-kinase (PtdIns-3′-kinase) correlates very well with the stimulated exit from G_0 into the cell cycle (reviewed by Cantley et al. 1991). Mutants of the receptors for PDGF and CSF-1 which have reduced coupling to the inositol lipid 3′-kinase activity also exhibit a reduced mitogenic response to added growth factor (Coughlin et al. 1989; Reedijk et al. 1990) whilst transformation defective mutants of MTAg, pp60$^{v\text{-}src}$ and pp160$^{gag\text{-}abl}$, also fail to associate with inositol lipid 3′-kinase activity. However, none of these studies constitutes evidence of a *necessity* for the inositol lipid 3′-kinase activity in mitogenic signalling.

4 Evidence for Multiple Sources of DG in Mitogen-Stimulated Cells

Whilst the hydrolysis of inositol lipids is implicated in the mediation of early mitogenic events, various observations suggest that the DG/PKC arm

of the signalling pathway may play a more important and sustained role than the transient Ins(1,4,5)P_3-mediated increases in $[Ca^{2+}]_i$. Thus, in bombesin-stimulated Swiss 3T3 cells the PKC-mediated increase in pH_i is sustained (Bierman et al. 1990) and serves as a major determinant of growth factor-stimulated S6 kinase activity whilst phosphorylation of the acidic 80-kDa MARCKS protein persists long after Ins(1,4,5)P_3-stimulated $[Ca^{2+}]_i$ elevation has declined (Takuwa et al. 1991). In BALB/c 3T3 cells micro-injection of DG but not Ins(1,4,5)P_3 is co-mitogenic with insulin (Suzuki-Sekimori et al. 1989). Furthermore, a number of growth factors which do not stimulate inositol lipid hydrolysis are still able to increase cellular DG content or activate a PKC cascade, e.g. CSF-1 (Immamura et al. 1990) and embryonal carcinoma-derived growth factor (Mahadevan et al. 1987). Such observations suggest that growth factors are able to generate DG from a source other than inositol lipids.

Growth factors of the RGE type, e.g. α-thrombin and bombesin, elicit very similar changes in inositol phosphate accumulation in cultured IIC9 and Swiss 3T3 fibroblasts. In both cases the generation of Ins(1,4,5)P_3 or inositol trisphosphate ($InsP_3$) is transient; mass analysis reveals that Ins(1,4,5)P_3 levels peak at 5 or 10 s before returning to prestimulated levels by 30 s (Cook et al. 1990). In contrast, mass analysis reveals that DG levels are increased in a sustained manner. Furthermore, the increase in DG mass is often biphasic; the first phase coincides with increases in Ins(1,4,5)P_3 whilst from approximately 30 s onwards DG levels rise again in a second sustained phase (Wright et al. 1988; Fukami and Takenawa 1989; Cook et al. 1990) which is maintained for up to 60 min and 4 h (Takuwa et al. 1989, 1991). Similar observations have been made in vasopressin-stimulated REF-52 cells (Huang and Cabot 1990b) and seem to be typical of those mitogens which couple to PIC via the guanine nucleotide regulatory protein G_p. The fact that the initial formation of DG is often discernable as a distinct peak which rapidly subsides suggests that there is likely to be rapid removal of 1-stearoyl-2-arachidonoyl glycerol by DG kinase (DGK; MacDonald et al. 1988) whilst the slower increase suggests a "second phase" of DG formation rather than the slower regulated removal of DG derived from PtdIns(4,5)P_2 hydrolysis. Indeed, PtdIns(4,5)P_2 hydrolysis is generally a rapidly desensitizing event for RGE-type mitogens; mass analysis reveals that in Swiss 3T3 cells bombesin-stimulated Ins(1,4,5)P_3 formation is desensitized to further agonist challenge within 30 s of the initial stimulus (Cook et al. 1990).

A second major piece of evidence for DG being derived from a source other than the inositol lipids comes from the observation that addition of phorbol esters, e.g. PMA, to a variety of cell types increases cellular DG content (e.g. Takuwa et al. 1987; Huang and Cabot 1990a; Martin et al.

1990). In this instance the DG cannot be derived from inositol lipids since it is well documented that PMA does not stimulate, but rather, inhibits inositol lipid hydrolysis (e.g. Brown et al. 1987). Our own studies in Swiss 3T3 cells (Cook et al. 1991) confirm that PMA stimulates a sustained increase in DG mass (Takuwa et al. 1987) that mimics, in part, the second phase of the response to bombesin. However, this occurs over the same dose range at which PMA inhibits bombesin-stimulated inositol phosphate accumulation (Brown et al. 1987; Cook and Wakelam 1991). Thus, phorbol esters can effectively dissociate DG formation from inositol lipid hydrolysis.

Finally, there are a number of growth factors of the RTK type which are able to increase DG levels and activate PKC without stimulating inositol phosphate accumulation. Stimulation of many cells with c-*sis* or PDGF BB, acting at either the α- or β-type receptors, results in the formation of both Ins(1,4,5)P_3 and DG, but it has recently been shown that stimulation with the AA isoform of PDGF, acting at the α receptor, results in the generation of DG and activation of PKC in the absence of inositol phosphate accumulation (Block et al. 1989; Sachinidis et al. 1990). Furthermore, PDGF can stimulate the formation of DG in the absence of inositol phosphate accumulation in IIC9 fibroblasts (Pessin et al. 1990). In Swiss 3T3 cells PDGF BB (c-*sis*) stimulates formation of Ins(1,4,5)P_3 but the net formation of DG mass greatly exceeds that of Ins(1,4,5)P_3, which might suggest that DG is derived from additional sources (Plevin et al. 1991 a). Thus it appears that formation of DG and activation of PKC are common responses to PDGF and can in some circumstances be dissociated from inositol lipid hydrolysis at the receptor level. Different receptor subtypes coupling to different sources of DG may also explain why fibroblast growth factor (FGF) is able to activate inositol lipid hydrolysis and PKC in one clone of Swiss 3T3 cells (Brown et al. 1989) whilst in another clone activation of PKC occurs in the absence of inositol phosphate accumulation (Nanberg et al. 1990). It is now apparent that there are a number of distinct FGF receptors (Keegan et al. 1991) and it will be interesting to see if these different receptor types, independently expressed, can couple to different DG-generating signal transduction pathways.

The case of EGF is more problematic since its ability to stimulate inositol lipid hydrolysis varies between cell types (see above). In those cell lines where EGF stimulates inositol lipid hydrolysis (e.g. A431 cells) this will necessarily to accompanied by the formation of DG. However, it is now apparent that EGF can stimulate increases in DG mass in IIC9 cells (Wright et al. 1988, 1990) and in Swiss 3T3 cells (Cook and Wakelam 1991) in the absence of inositol lipid hydrolysis. Whether these responses are associated with activation of PKC is a subject of continued debate (Isacke

et al. 1986; Rodriguez-Pena and Rozengurt 1986; Kazlauskas and Cooper 1988).

In addition to these specific examples there are a number of reports which strongly suggest that growth factors can activate a DG/PKC signalling pathway in the absence of inositol lipid hydrolysis (see above). Thus generation of DG and/or activation of PKC appear to be common events following stimulation by a variety of growth factors; in some cases this will be by the "classical" pathway of inositol lipid hydrolysis but in other instances will occur independently of stimulated polyphosphoinositide metabolism.

5 Alternative Pathways of DG Formation?

5.1 Hydrolysis of PtdIns

PLC-catalysed hydrolysis of PtdIns, PtdIns(4)P and PtdIns(4,5)P_2, or indeed any glycerophospholipid, can conceivably give rise to net increases in DG. In vascular smooth muscle cells (VSMC) Griendling et al. (1986) have argued that hydrolysis of PtdIns(4,5)P_2 is the primary but transient event which is followed by a later Ca^{2+}-dependent hydrolysis of PtdIns to yield InsP and DG. They also speculate that activation of PKC following PtdIns(4,5)P_2 hydrolysis may serve to activate PtdIns hydrolysis; however, this seems unlikely since activation of PKC by PMA or DG analogues does not stimulate but rather inhibits inositol hydrolysis (Brown et al. 1987).

A more convincing argument for sustained hydrolysis of PtdIns comes from the studies of Imai and Gershengorn (1986) in thyrotropin-releasing hormone (TRH)-stimulated GH_3 pituitary cells. In cells labelled with ^{32}Pi for only 1 min and then challenged with TRH the increase in labelling of PtdIns(4,5)P_2 was transient, lasting at most 2 min, whereas the labelling of PtdIns and PtdOH was sustained. Furthermore, if GH_3 cells were stimulated with TRH for 4 min prior to addition of ^{32}Pi there was a sustained increase in the labelling of PtdIns and PtdOH without further labelling of PtdIns(4,5)P_2, suggesting that at 4 min PtdIns(4,5)P_2 hydrolysis and resynthesis was complete whereas PtdIns hydrolysis continued.

In a variety of stimulated cell lines accumulation of Ins(1,4,5)P_3 is transient, whereas in the presence of LiCl there is the sustained accumulation of InsP for up to 1 h. In the light of the work of Imai and Gershengorn (1986) it is possible that this represents sustained PtdIns hydrolysis. However, the high activity of Ins(1,4,5)P_3 5′-phosphatase and

3′-kinase may also explain why $Ins(1,4,5)P_3$ levels rapidly decline; thus, the return of $Ins(1,4,5)P_3$ levels to basal or near basal may represent a new "steady state" where stimulated formation is effectively matched by rapid removal. Hence, in the presence of LiCl the prolonged accumulation of InsP may reflect continued $PtdIns(4,5)P_2$ hydrolysis, albeit at a reduced rate, and the shunting of inositol moieties from $Ins(1,4,5)P_3$ to InsP. The prolonged accumulation of InsP indicates that PLC-catalysed inositol lipid hydrolysis does continue and this will necessarily result in DG accumulation; whether $PtdIns(4,5)P_2$ or PtdIns is the major substrate for PIC at these later times remains to be established.

5.2 Hydrolysis of PtdCho and Other Phospholipids

The source of sustained DG formation had been ascribed to hydrolysis of PtdIns (Griendling et al. 1986), but recent evidence now implicates the hydrolysis of PtdCho. In mitogenic systems the most thorough and definitive studies have been by Raben and co-workers (Pessin and Raben 1989; Pessin et al. 1990) who separated derivatised DGs from IIC9 cells by gas-liquid chromatography (g.l.c.) on the basis of the fatty acyl composition and assayed the individual molecular species by mass spectrometry. By comparing the profiles with that from DGs derived by PLC-catalysed cleavage of cellular PtdIns, PtdCho, PtdEtn and PtdSer they were able to show that only the DG produced in the initial phase of the response to α-thrombin was derived from inositol lipids (typically 1-stearoyl-2-arachidonoyl). At 5 and 60 min, the sustained phase of DG formation, the acyl profile of DGs produced by α-thrombin stimulation was closest to that derived from PtdCho. In addition the sustained formation of DG stimulated by EGF and PDGF in this system, in the absence of inositol phosphate accumulation, also exhibited a molecular species profile which was closest to PtdCho. Interestingly, the molecular species profile of sustained DG formation in response to EGF, PDGF and α-thrombin was not identical to PtdCho but did greatly resemble each other suggesting the possibility of distinct hormone-sensitive pools of PtdCho enriched in particular fatty acids or selective diglyceride metabolism (Pessin et al. 1990).

In addition to these studies, a number of growth factors stimulate the accumulation of choline (Cho) and phosphocholine (ChoP) in cells prelabelled with $[^3H]$Cho as well as or instead of stimulating the accumulation of inositol phosphates (e.g. Besterman et al. 1986b; Muir and Murray 1987; Cook and Wakelam 1989; Price et al. 1989; Larrodera et al. 1990; Wright et al. 1990; Imamura et al. 1990) suggesting hydrolysis of PtdCho by a PLD- or PLC-catalysed pathway. In many of these cases the kinetics

of Cho/ChoP release are very similar to those for DG formation. Furthermore, a number of reports have shown that the use of isotopically labelled fatty acids such as oleic acid, myristic acid and palmitic acid allows the preferential labelling of cellular PtdCho over all other classes with negligible incorporation into inositol lipids. The stimulated formation of radiolabelled DG under these conditions is further evidence for DG being derived from a non-inositide source, probably PtdCho.

6 PdtCho Hydrolysis: PLC or PLD?

Two pathways for DG formation from PtdCho have been proposed (see Exton 1990; Billah and Anthes 1990); the direct hydrolysis by PLC to give DG and ChoP or a coupled pathway where PLD activity gives rise to PtdOH and Cho and the PtdOH is then dephosphorylated by PtdOH phosphohydrolase (PPH) (PLD/PPH pathway). However, the release of, e.g. [^{3}H]Cho or [^{3}H]ChoP does not serve as a definitive assay for PtdCho-PLD or -PLC since the products of each reaction may be readily interconvertible by the actions of choline kinase (or ChoP phosphatase), DGK and PPH (see Billah and Anthes 1990). Definitive assignment of PLD activity in whole cells can be achieved by two protocols; first, the formation of [^{32}P]PtdOH in cells labelled with [^{32}P]PtdCho so as not to label the cellular ATP pool and second, the use of primary alcohols to substitute for water in the phosphatidyltransferase reaction of PLD giving rise to the corresponding phosphatidylalcohol. Moreover, since the phosphatidylalcohols are poor substrates for PPH (Metz and Dunlop 1991) the use of primary alcohols allows intervention in a PLD/PPH pathway of DG formation (e.g. Bonser et al. 1989). Figure 2 outlines the transphosphatidylation activity of PLD and describes the coupled pathway. Formation of [^{32}P]ChoP under conditions where the cellular ATP pool is not labelled will define PLC activity. An alternative is to assay the mass of Cho and ChoP formed; this should allow an estimation of whether ChoP formation can be accounted for solely by choline kinase activity or not. In common with Billah and Anthes (1990) we feel that these criteria are the minimum with which one can reliably define a PtdCho-PLD activity.

With these caveats in mind, a number of growth factors and mitogenic agents have been shown to stimulate PtdCho hydrolysis by a PLD pathway. Phorbol ester-stimulated PLD activity has been reported in a variety of cell types and appears to be an almost ubiquitous phenomenon (reviewed by Billah and Anthes 1990). PDGF is able to stimulate PLD transferase activity in NIH3T3 (Ben-Avi and Liscovitch 1989) and Swiss 3T3 (Plevin et al.

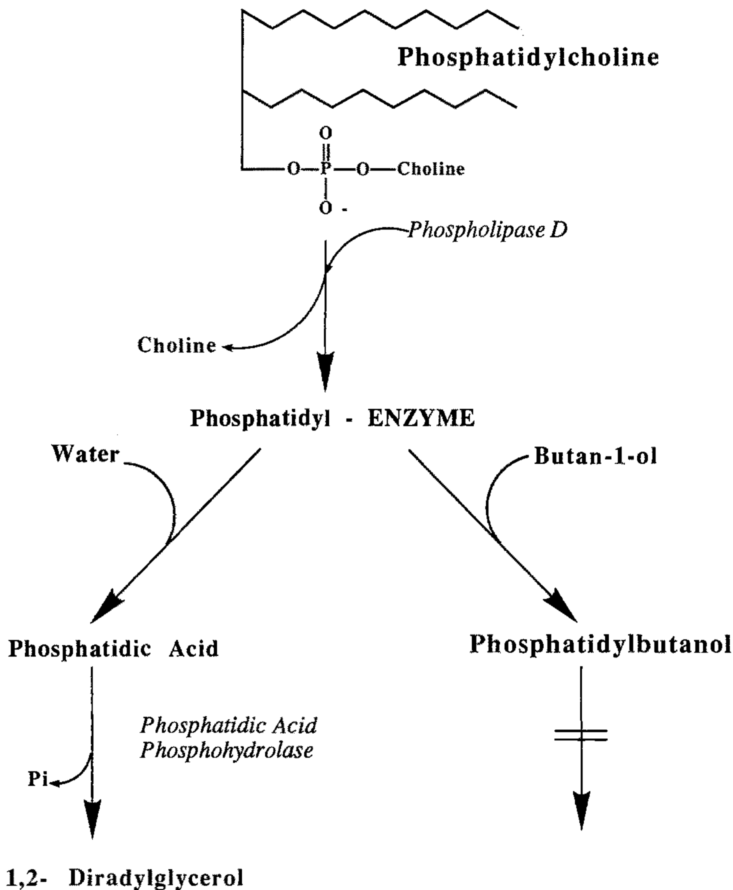

Fig. 2. PLD activity. PLD catalyses a transphosphatidylation reaction which can utilise water or a short chain aliphatic alcohol as a nucleophilic acceptor. The former reaction generates phosphatidic acid which can be hydrolysed to generate 1,2-diglyceride by the action of phosphatidic acid phosphohydrolase

1991 a) whilst EGF is able to stimulate PtdCho-PLD acitivity as measured by the formation of phosphatidylalcohols in Swiss 3T3 cells (Cook and Wakelam 1991). Both α-thrombin and bombesin are able to stimulate the formation of phosphatidylalcohols with identical kinetics to [^{3}H]Cho release in IIC9 and Swiss 3T3 cells; similar results are obtained with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and vasopressin (Huang and Cabot 1990b; Cook and Wakelam 1991). Despite this the role of PLD in the sustained formation of DG is unclear. Bonser et al. (1989) have shown that primary

alcohols can be used to assess the role of a PLD/PPH pathway in DG formation in the human neutrophil; the inhibition of DG and O_2^- formation by butan-1-ol indicates that PLD/PPH-derivated DG is required for the oxidative burst in neutrophils. In IIC9 fibroblasts both EGF and α-thrombin stimulate sustained increases in DG and [^{3}H]Cho and [^{3}H]ChoP and are both able to stimulate PLD transferase activity in the presence of ethanol. However, ethanol does not inhibit DG formation by either agonist suggesting that whilst PLD is activated it does not serve as the pathway of DG formation (D.M. Raben, personal communication). In Swiss 3T3 cells bombesin and PMA stimulate PLD transferase activity (assayed in the presence of butan-1-ol) but this seems to play a quite different role in DG formation for the two agonists. Butan-1-ol inhibits up to 70% of PMA-stimulated DG formation suggesting a major role for a PLD/PPH pathway whereas bombesin-stimulated DG formation is inhibited by at most 30% in the presence of butan-1-ol (Cook et al. 1991). Since PMA-stimulated PLD activity is sustained whereas bombesin-stimulated PLD activity is rapidly desensitized these differences may reflect the duration of PLD activation. Indeed it is apparent in a number of cases (e.g. α-thrombin, EGF and bombesin) that activation of PLD is transient, being largely complete after 2–5 min of stimulation. Thus, even when PLD is activated it may make only a relatively small or transient contribution to sustained DG formation. Martinson et al. (1990) have recently shown a similar transient activation of PLD in carbachol-stimulated 1321N1 astrocytoma cells and speculate that the sustained elevation of DG may be due to the slow dephosphorylation of PtdOH by PPH. However, studies by Wright (1988) suggest that PPH is unlikely to be a limiting component in a coupled PLD/PPH pathway. The recent description of sphingosine as a PKC-independent inhibitor of PPH may provide a tool for addressing this anomaly.

If PLD activity has ceased within 2–5 min then what is the source of sustained increases in DG after 5 min? Those responses where both Cho and ChoP are increased may indicate that both PLD and PLC are activated; in bombesin-stimulated Swiss 3T3 cells the rise in ChoP occurs after the rise in Cho suggesting that PLD activity may be the first event in PtdCho hydrolysis followed by PLC (Cook and Wakelam 1989). However, by the criteria described above we are not aware, to date, of any study which has defined a mitogen-activated PtdCho-PLC activity. The study of Larrodera et al. (1990) is of interest since they were only able to detect the formation of [^{3}H]ChoP in PDGF-stimulated Swiss 3T3 cells; however, the first stimulated time point was 2 h after addition of PDGF making it impossible to correlate with an early receptor-mediated event. Further, in Swiss 3T3 cells the release of [^{3}H]Cho and activation of PLD transferase

activity have been demonstrated within minutes of PDGF stimulation (Plevin et al. 1991 a). It is possible that slower increases in ChoP may represent PLC activity, but this remains to be rigorously defined; increased in [^{3}H]ChoP after 2 h of stimulation cannot be considered definitive assays for PLC as outlined above.

In addition, however, we feel that hydrolysis of inositol lipids may continue to make some contribution to sustained DG formation even after Ins(1,4,5)P_3 levels have declined. Imai and Gershengorn (1986) have previously shown that in TRH-stimulated GH_3 pituitary cells PtdIns(4,5)P_2 hydrolysis is a transient event which is followed by the sustained hydrolysis of PtdIns. In the presence of LiCl the bombesin-stimulated accumulation of total inositol phosphates and InsP occurs at two distinct rates in Swiss 3T3 cells (Cook et al. 1991). Accumulation is rapid from 0–1 min and reduced in rate up to eight fold thereafter but continues at this rate for at least 30 min. Whether this reflects continued, albeit reduced, PtdIns(4,5)P_2 hydrolysis or sustained hydrolysis of PtdIns remains to be seen. This may still be consistent with the molecular species analysis of Pessin et al. (1989, 1990); whilst polyphosphoinositides are characterised by 1-stearoyl-2-arachidonoyl molecular species total cellular PtdIns exhibits a more heterogeneous profile of fatty acids which contribute to sustained generation of DG by RGE-type growth factors. Thus, the sustained generation of DG may be a composite of transient PLD activity, a possible PtdCho-PLC and continued inositol lipid hydrolysis.

7 Regulation of PLD

Studies in Swiss 3T3 cells suggest that for RGE-type mitogens the activation of PtdCho-PLD is secondary to inositol lipid hydrolysis and activation of PKC (Cook and Wakelam 1989, 1991). In the case of bombesin the onset of PLD activity is kinetically downstream of the first phase of DG/Ins(1,4,5)P_3 formation (Cook and Wakelam 1989; Cook et al. 1990) whilst activation of PLD is abolished in cells which have been depleted of cellular PKC activity by chronic treatment with high doses of phorbol ester. Similar observations have been made in adrenal glomerulosa cells (Liscovitch and Amsterdam 1989), smooth muscle cells and endothelial cells and suggests that PKC plays a major role in regulating PLD. These studies are supported by the use of PKC inhibitors such as staurosporine (Martin et al. 1990), H7 (Liscovitch and Amsterdam 1989) and Ro-31-8220 (Wakelam et al. 1991) which inhibit agonist-stimulated PLD activity. However, in many cases the inhibition of agonist-stimulated PLD (not phorbol

ester-stimulated) by PKC inhibitors is only partial whereas the effect of PKC down-regulation is essentially maximal. In Swiss 3T3 cells bombesin-stimulated PLD acitivity is completely inhibited by PKC down-regulation but is only inhibited by 50% by Ro-31-8220 at concentrations which completely inhibit phorbol ester-stimulated PLD activity. This suggests that the total abolition of bombesin-stimulated PLD activity by PKC down-regulation is to some extent artefactual and that PLD activity per se is compromised by prolonged exposure of cells to phorbol esters. Whilst the effect of this treatment upon total PKC activity is well characterised in Swiss 3T3 cells it remains unclear if different PKC isoforms are differentially sensitive to prolonged phorbol ester treatment or whether other proteins are also susceptible to this treatment.

Evidence for a role for Ca^{2+} in regulating PLD varies markedly between cell types. In human erythroleukaemia cells (Halenda and Rehm 1990) and neutrophils (Agwu et al. 1989) Ca^{2+} appears to play the major role in regulating PLD whereas in Swiss 3T3 cells the role of Ca^{2+} appears to be subordinate to that of PKC where even A23187-stimulated PLD activity is inhibited by the PKC inhibitor Ro-31-8220 (Wakelam et al. 1991). Combination of the Ro-31-8220 and ethylene glycol tetra-acetic acid (EGTA) treatments is less than additive in inhibiting bombesin-stimulated PLD activity; the residual PLD activity under these conditions suggests that other elements are involved in the regulation of PLD. It still remains for there to be a thorough assessment of the role of Ca^{2+} in the regulation of PLD including the relative roles of Ca^{2+} entry and Ca^{2+} mobilisation from intracellular stores.

A role for a guanine nucleotide-binding protein in regulating PLD activity has been suggested by reports of stimulation of PLD transferase activity by non-hydrolysable analogues in homogenates from HL-60 cells (Anthes et al. 1989) and by the stimulated release of Cho from hepatocytes (Bocckino et al. 1987). Similar observations have been made in cell-free preparations from rat brain (Kobayashi and Kanfer 1987) and in permeabilised endothelial cells (Martin and Michaelis 1989). In cell-free preparations of HL-60 cells Ca^{2+} guanosine 5′-(γ-thio)-triphosphate (GTPγS) can stimulate PLD activity only in the intact cell homogenate, not in the isolated cytosol or membrane fractions (Anthes et al. 1991). The authors conclude that PLD activity exhibits an essential requirement for a heat labile factor (protein) whose activity depends upon GTPγS. Whether this is a heterotrimeric GTP-binding protein analogous to G_s, G_p or G_i remains to be seen. In a variety of permeabilised cells agonist will greatly accelerate GTPγS-driven PIC activity consistent with receptor-driven guanine nucleotide exchange being a rate-limiting step in activation of inositol lipid hydrolysis. Such experiments have yet to be performed on

agonist-stimulated PLD activity. Thus, whilst there is evidence for a guanine nucleotide-stimulated PLD activity in some cells (notably HL-60 cells) it remains to be defined as a classical receptor-"G_d"-PLD-coupled cascade.

One intriguing possibility is that a small-molecular-weight GTP-binding protein may regulate PLD activity. *ras*-transformed fibroblasts exhibit elevated levels of ChoP and ethanolamine phosphate, and this has been ascribed to increased PtdCho-PLC activity (Lacal et al. 1987). However, this increase in ChoP may be due to enhanced choline kinase activity in *ras*-transformed cells (Macara 1989; Wakelam et al. 1990) which could in turn be due to enhanced levels of Cho; these studies serve to underline the problems in studying basal levels of second messengers in chronically transformed cells. In NIH3T3 cell membranes GTPγS clearly stimulates the formation of both Cho and $InsP_3$ but it does so to the same extent in both control and c-Ha-*ras*-transformed NIH3T3 cell membranes (Quilliam et al. 1990). Thus, although there is evidence for GTP-binding protein regulation of PIC and PLD, there is no convincing evidence that *ras* regulates either phospholipase.

There is little evidence for a direct receptor-coupled PLD activity. In the case of RGE-type mitogens such as bombesin the onset of PLD activity is kinetically downstream of stimulated Ins(1,4,5)P_3 (Cook and Wakelam 1989; Cook et al. 1990) and this is consistent with the abolition of bombesin-stimulated PLD activity following chronic treatment with phorbol esters or PKC inhibitors (Cook and Wakelam 1989, 1991). The case of α-thrombin is of interest since the second phase of DG formation, from PtdCho (Pessin and Raben 1989), occurs over a much lower dose range than the prior inositol lipid-derived DG formation (Wright et al. 1988) suggesting that the two responses might be mediated by pharmacologically distinct receptor populations. However, it remains to be seen if the dose dependence for α-thrombin-stimulated PtdCho hydrolysis is distinct from stimulated inositol phosphate accumulation.

EGF-stimulated PLD activity occurs in the absence of inositol lipid hydrolysis in IIC9 cells and Swiss 3T3 cells (Wright et al. 1988, 1990; Cook and Wakelam, submitted) and in the latter case this correlates with the lack of effect of PKC inhibitors; thus it seems unlikely that PLD activity is regulated by activation of PKC consequent with inositol lipid hydrolysis. Tyrphostins inhibit EGF-stimulated PLD activity in Swiss 3T3 cells (Cook and Wakelam 1992). In a variety of other systems such results have been taken to show that the EGF RTK activity couples directly to the relevant effector system, but all they really show is that a tyrosine phosphorylation event is required for activation of PLD; the site of phosphorylation awaits to be defined.

The availability of receptor cDNAs allows expression-defined agonist receptors in naive cell lines (i.e. those cell lines which do not normally possess a given receptor). In seeking to address the role of inhibition of adenylyl cyclase in mitogenic signalling MacNulty et al. (1992) transfected the platelet α_2-adrenergic receptor into Rat-1 fibroblasts. As well as conferring ligand-dependent inhibition of adenylyl cyclase activity upon the transformants it was found that α_2 agonists were able to stimulate PLD activity but not inositol lipid hydrolysis (MacNulty et al. 1992). Both adenylyl cyclase activity and PLD were abolished in P-tox-treated cells suggesting that both responses are activated by G_i- or G_o-like guanine nucleotide regulatory proteins. Furthermore, this raises the possibility that combination of both signalling pathways is *sufficient* for a mitogenic response.

8 The Role of DG and PtdOH Derived from PtdCho Hydrolysis

8.1 The Role of Sustained Increases in DG

The most obvious role for sustained DG formation, from PtdCho and/or inositol lipids, is to sustain PKC activation which may thereby constitute a potent mitogenic signal. It is now apparent that PKC represents a large family of enzyme isoforms with distinct activation requirements in vitro and perhaps distinct substrate specificities (Kikkawa et al. 1989; Parker et al. 1989; Schaap and Parker 1990). Thus, whilst PKC-α is most sensitive to Ca^{2+} and 1-stearoyl-2-arachidonoyl glycerol, the major species derived from polyphosphoinositide hydrolysis, β-I and β-II exhibit substantial activity without added Ca^{2+} in the presence of DG and phospholipid and PKC-γ can be activated by the micromolar range of free arachidonic acid in the absence of Ca^{2+}, DG or phospholipid. In addition, there is a rapidly expanding subfamily of PKC isoforms which lack the C_2 regulatory domain implicated in interactions with Ca^{2+} (reviewed by Parker et al. 1989). These include PKC-δ, -ε, -ε', -ζ (Ono et al. 1987), nPKC (Ohno et al. 1988) and PKC-L (Bacher et al. 1991). At least three of these isoforms, PKC-ε, nPKC and PKC-L, exhibit considerable catalytic activity and phorbol ester binding in the absence of added Ca^{2+} (Ohno et al. 1988; Schaap and Parker 1990; Bacher et al. 1991) whilst PKC-δ and -ε are significantly activated in the absence of DG (Ono et al. 1988). Furthermore, PKC-ε exhibits a distinct substrate specificity in vitro compared to -α, -β, and -γ particularly against "classical" substrates such as histone IIIS (Schaap and Parker 1990). Consequently, generation of DG from PtdCho in the absence of increased $Ins(1,4,5)P_3$ and $[Ca^{2+}]_i$ may serve to activate distinct species

of PKC with their own substrate specificity. Thus, different phases of DG formation and PKC activation may serve to widen the repertoire of intracellular signals generated by a given growth factor. In this respect it will be of interest to see if different molecular species of DG, derived from PtdCho hydrolysis, are able to activate various isoforms of PKC to differing degrees. The apparent variation in substrate specificity of different PKC isoforms, if reflected in vivo, also suggests that the use of marker phosphoproteins (e.g. the 80-kDa MARCKS protein) as assays of PKC activation should be treated with caution; lack of phosphorylation of the 80-kDa protein may not necessarily mean that no isoform of PKC is being activated (viz. the debate as to whether EGF is able to activate PKC in Swiss 3T3 cells).

The ability of PtdCho-derived DGs to activate PKC remains a subject of debate. In smooth muscle cells PDGF-AA, acting at the α-type receptor, is able to increase DG levels and activate PKC in the absence of Ins(1,4,5)P_3 formation (Block et al. 1989; Sachidinis et al. 1990) and it is well documented that PDGF can stimulate DG formation derived from PtdCho (Pessin et al. 1990). In bombesin-stimulated Swiss 3T3 cells both DG accumulation and phosphorylation of the MARCKS protein are sustained for at least 4 h (Takuwa et al. 1991) and the biphasic increase in DG (Cook et al. 1990) correlates well with the biphasic increase in pH_i by the PKC-activated Na^+/H^+ antiporter (Bierman et al. 1990). In contrast, Leach et al. (1991) have shown that whilst PtdCho-derived DG is able to activate PKC in vitro, in α-thrombin-stimulated IIC9 fibroblasts activation of PKC is only associated with the first transient phase of DG formation derived from inositol lipid hydrolysis and not that derived from PtdCho. At low concentrations α-thrombin remains mitogenic, but is no longer able to activate PtdIns(4,5)P_2 hydrolysis or activation of PKC suggesting that activation of PKC is not required for the mitogenic response to this growth factor. There may be a number of reasons for these differing reports of the role of sustained DG formation in activating PKC. PtdCho-derived DGs may be formed in a distinct cellular compartment with no access to PKC; in this respect it is yet to be confirmed where in the cell PtdCho hydrolysis takes place. If DG derived from PtdCho activates distinct isoforms of PKC this response will only be manifest in cells expressing the given isoform; Leach et al. (1991) were only able to screen IIC9 cells for the presence of PKC-α, -β and -γ. Thus, the inability of α-thrombin to stimulate phosphorylation of the MARCKS protein may be due to lack of the relevant isoform in IIC9 cells or the MARCKS protein being a poor substrate for particular isoforms.

An alternative and simpler explanation is that DG derived from PtdCho does not activate PKC. Whether sustained DG formation performs some

other function involved in the prereplicative phase remains to be seen since it correlates well with the mitogenic response to α-thrombin (Wright et al. 1988, 1990; Pessin et al. 1989, 1990; Leach et al. 1991) and sustained early signals in Swiss 3T3 cells (Takuwa et al. 1991). Gonzatti-Haces and Traugh (1986) have previously reported that DG and phospholipid will activate protease-activated kinase II (PAK II) in the absence of Ca^{2+} whilst Burch (1988) has reported that DG will activate phospholipase A (PLA_2) from Swiss 3T3 cells under conditions which do not support PKC activity. Certainly a number of other proteins contain cysteine-rich sequences homologous to PKC including the Raf-1 serine/threonine kinase and the human glucocorticoid receptor, and the description of novel phorbol ester receptors such as *n*-chimaerin (Ahmed et al. 1990 and references therein) suggests that DG/PMA may exert effects independently of PKC.

8.2 A Role of PtdOH as a Mitogenic Messenger?

Activation of PLD appears to be a common response to a variety of mitogenic and nonmitogenic stimuli but may not always function as a pathway for DG formation (Huang and Cabot 1990b; Raben et al. personal communication; Cook and Wakelam, in preparation; Plevin and Wakelam, in preparation) suggesting that PtdOH may serve other distinct functions in addition to that of a precursor of DG via PPH.

Various roles have been proposed for PtdOH. Reports of a Ca^{2+} ionophore function (Putney et al. 1980) have remained unsubstantiated, but in neutrophils the kinetics of PtdOH formation, produced largely by a PLD pathway (Cockcroft 1984; Billah et al. 1989), correlate with enzyme secretion (Cockcroft 1984) suggesting a role for PtdOH in membrane fusion and secretion events. Certainly the membrane fusogenic properties of PtdOH and DG are well documented (reviewed by Wakelam 1988).

The exogenous addition of PtdOH and particularly its lyso-derivative, lyso-PtdOH, to fibroblast and nonfibroblast cells results in the hydrolysis of polyphosphoinositides, activation of PKC, inhibition of adenylyl cyclase and stimulation of DNA synthesis (Moolenaar et al. 1986; Murayama and Ui 1987; Van Corven et al. 1989; Plevin et al. 1991b) suggesting that the two agents may act as growth factors. It seems likely that lysophosphatidic acid (lyso-PtdOH) is more potent than PtdOH and that some of the reported effects of PtdOH may be due to contamination of commercial preparations with lyso-PtdOH (Jalink et al. 1990). Whether PtdOH and lyso-PtdOH produced by intracellular signal pathways actually leave the cell membrane and act as auto- and paracrine mitogens remains unclear; such speculation will await the identification of receptors for these molecules.

In the context of mitogenic messenger properties PtdOH and arachidonic acid are both able to inhibit p21^{ras}GTPase-activating protein (*ras* GAP; Tsai et al. 1989) and stimulate the putative *ras*-GTPase-inhibiting protein (*ras* GIP; Tsai et al. 1990) in vitro. As described previously, the net effect of both of these events, should they occur in vivo, would be to greatly increase the amount of p21^{ras} in its active GTP-bound state. Both PDGF and EGF have been shown to activate *ras* by increasing the ratio of p21^{ras}GTP/GDP (Satoh et al. 1990a, b). Since EGF and PDGF are also able to activate PLD it is tempting to speculate that the PtdOH/DG produced may be involved in the activation of *ras* by virtue of their effects upon GAP and GIP. However, it seems likely that the ability of EGF and PDGF to phosphorylate GAP upon tyrosine residues (Molloy et al. 1989; Kaplan et al. 1990; Ellis et al. 1990) will also play a major role, though what effect this phosphorylation has upon GAP function is not known.

There are recent reports that PtdOH may actually activate kinases in a manner analogous to DG. Epand and Stafford (1990) have shown that PtdOH will support a PKC activity which is inhibited by Ca^{2+} in a dose-dependent manner. In addition a preliminary report from Bocckino and Exton (1990) suggests that PtdOH can stimulate a distinct pattern of protein phosphorylation from that induced by DG in an in vitro assay using rat liver cytosol as a source of kinase activity. Thus, it seems likely that PtdOH may serve a role as a second messenger in its own right with perhaps diverse intracellular and extracellular targets, as well as a precursor of DG, and further research into its possible function is clearly warranted.

Acknowledgements. Experimental work in MJOW's laboratory is supported by grants from the Medical Research Council (UK), the Wellcome Trust, the British Heart Foundation and the Cancer Research Campaign.

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Rev. Physiol. Biochem. Pharmacol., Vol. 119
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Lysophosphatidic Acid: A Bioactive Phospholipid with Growth Factor-Like Properties

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1 Introduction

Increasing attention is currently being focussed on understanding signal transduction by growth factors, the prototypes of which are platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) (Williams 1989; Ullrich and Schlessinger 1990). Growth factors initiate their action by binding to specific high-affinity receptor molecules on the cell surface, thereby triggering a myriad of early biochemical changes that ultimately may lead to altered gene expression, DNA replication, and cell division, but to link these early changes with the ultimate stimulation of DNA synthesis, occurring many hours later, remains a central problem in the growth factor field. Although many growth factors and their receptors have been identified, cloned, and biochemically characterized, the signal transduction pathways that culminate into replicative DNA synthesis and cell proliferation are poorly understood. The identification of novel mitogens acting on the cell surface and the elucidation of their mechanism of action may help to unravel the complex "mitogenic signaling network", where multiple signal cascades often act in parallel and similar responses may be produced by different means.

In recent years, membrane phospholipids have attracted a great deal of interest in studies on cellular signaling and growth control. It has long been realized that the variety of cellular phospholipids, as well as their metabolism, is highly complex. While a major function of phospholipids is to form bilayer membranes, the breakdown products of several plasma membrane phospholipids appear to act as signal molecules, i.e., as intracellular second messengers or as agonists that modulate cell function. The best known examples of phospholipid-derived signal molecules include diacylglycerol, inositol trisphosphate (IP_3) and prostaglandins, which are all rapidly generated when cells are stimulated by certain hormones or growth factors.

The simplest naturally occurring phospholipids, phosphatidic acid (PA or diacylglycerol-3-phosphate) and lysophosphatidic acid (LPA or monoacylglycerol-3-phosphate), are of particular interest in that they not only are critical intermediates in de novo lipid biosynthesis and are rapidly produced in activated cells but also can stimulate cell proliferation when added to appropriate target cells in culture. Understanding of the growth factor-like action and the normal biological function of these simple, low-abundance phospholipids in normal and abnormal cell growth is obviously an important research goal.

The intention of this chapter is to summarize the current state of knowledge about the multiple biological effects of PA and LPA, with particular emphasis on their growth factor-like activity. The potent mitogenic

action of exogenous (L)PA raises many questions to which there are only partial answers as yet. How do these phospholipids act, what is their primary cellular target and what is their metabolic fate? What is the spectrum of biological activities of LPA and PA? Are these molecules released from activated cells to serve as mitogens for neighboring cells? In addition to addressing these points, a brief overview is given of the role of endogenous LPA and PA in de novo lipid biosynthesis and their rapid formation in activated cells.

2 PA and LPA: Biosynthesis and Cellular Metabolism

2.1 Role in De Novo Lipid Biosynthesis

LPA and PA are well-known precursors in the early steps of phospholipid biosynthesis (for review see Bishop and Bell 1988 and references therein). During early biosynthesis the starting point is glycerol-3-phosphate, which is acylated by acyl coenzyme A (acylCoA) to yield LPA. LPA is then further acylated by the same enzyme (glycerol-phosphate acyl transferase) to form PA. The fatty acyl chain attached to the C_1 position of the glycerol backbone is often saturated, whereas the one attached to C_2 is usually unsaturated.

PA is located at a branch point in de novo phospholipid synthesis. PA can be hydrolyzed by a specific phosphatase to give diacylglycerol, which is then used for the synthesis of more complex phospholipids such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE). PA can also serve as a precursor for the synthesis of acidic phospholipids such as phosphatidylinositol and phosphatidylserine. These de novo metabolic reactions occur mainly in the endoplasmic reticulum membrane. This is in marked contrast to the rapid generation of PA and diacylglycerol through the hydrolysis of preexisting phospholipids during receptor activation, which occurs at the cell surface.

2.2 Generation in Activated Cells

Considerable progress has been made in recent years in understanding the generation of phospholipid-derived signal molecules. It is now well established that many hormones, neurotransmitters and growth factors exert their immediate cellular effects, at least in part, through the hydrolysis of phosphatidylinositol-4,5-P_2 (PIP_2) to inositol-1,4,5-P_3 (IP_3), mediating

the release of stored Ca^{2+}, and 1,2-diacylglycerol (DG), activating one or more of the several isozymes of the serine/threonine kinase, protein kinase C (PKC). Many, if not all, of these agonist also stimulate the breakdown of PC through the action of either a specific phospholipase C (PLC), phospholipase D (PLD), or both, to yield DG and PA, respectively. Current evidence suggests that PC breakdown may be regulated by PKC, Ca^{2+}, and G proteins, depending on cell types and agonists. The main characteristics of these signaling systems have been the subject of several recent reviews (Berridge 1987; Nishizuka 1986; Exton 1990; Downes and Macphee 1990; Billah and Anthes 1990).

When cells are stimulated by agonists, the level of PA in the plasma membrane often rises rapidly. PA can be generated via phosphorylation of newly formed DG by DG kinase (Kanoh et al. 1990) or, more directly, through the action of a cellular phospholipase D acting on PC and perhaps also PE (Bocckino et al. 1987; Exton 1990). In the latter case, the level of PA often rises more rapidly and to a higher level than that of DG. Whatever its mechanism of formation, PA can function not only as a source of DG (the second messenger for PKC) and as a precursor for cytidine diphospho-diacylglycerol) (CDP-DG) in de novo synthetic pathways, but also as a potential signal molecule in its own right as will be discussed below.

While the rapid appearance of PA in stimulated cells is generally observed as a direct consequence of the receptor-linked activation of PLC and/or PLD, the generation of LPA during cell activation has been much less thoroughly examined. LPA (mainly $C_{16:0}$ and $C_{18:0}$) has been shown to accumulate rapidly in thrombin-stimulated platelets (Lapetina et al. 1981; Watson et al. 1985; Gerrard and Robinson 1989). The production of LPA may be secondary to the formation of PA through the action of a PA-specific phospholipase A_2 or, alternatively, through phosphorylation of monoacylglycerol generated by a DG lipase. A PA-specific phospholipase A_2 activity, which differs from the type that degrades PC and PE, has indeed been found in platelets (Billah et al. 1981) and it, therefore, seems reasonable to assume that LPA formation is of physiological significance. An outline of PA and LPA metabolism in stimulated cells is illustrated in Fig. 1.

3 LPA and PA as Mitogens

In addition to being rapidly produced in stimulated cells, PA and LPA can exert their own biological effects. In particular, exogenous PA and LPA

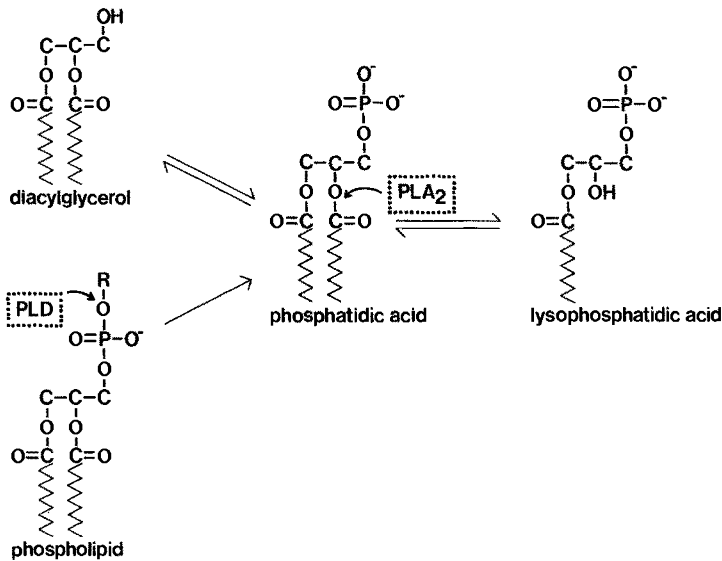

Fig. 1. Generation of PA and LPA from newly formed DG or preexisting phospholipids during cell activation. DG is rapidly formed via receptor-linked activation of PLC and is phosphorylated by DG kinase to yield PA. PA may also be formed through activation of PLD acting on PC and PE (*R* denotes phospholipid head group). PA may be hydrolyzed by a phospholipase A_2 (PLA_2) to LPA. The contribution of other pathways to generating LPA remains to be explored

stimulate DNA synthesis and cell division in fibroblasts and epithelial cells (Moolenaar et al. 1986; Siegmann 1987; Yu et al. 1988; Van Corven et al. 1989, 1992; Imagawa et al. 1989). Both LPA (1-oleoyl) and PA (1,2-dioleoyl) stimulate thymidine incorporation in Rat-1 cells with a half-maximal effect observed at about 15 μM and a saturating response at about 100 μM (Van Corven et al. 1992). Although PA often contains a few percent of contaminating LPA (Benton et al. 1982; Jalink et al. 1990), the observed dose-response curve of the mitogenic effect of PA cannot simply be explained by LPA impurities in the PA used. LPA is about as equally potent as EGF or 10% fetal calf serum in stimulating thymidine incorporation and cell division in Rat-1 fibroblasts. As with polypeptide growth factors, the mitogenic response to LPA requires long-term presence of the stimulus. When LPA is removed from the culture medium several hours after stimulation, the cells fail to enter S phase. The growth-stimulating activity does not require the presence of peptide growth factors: neither insulin nor EGF were found to act in a synergistic fashion with LPA (Van Corven et al. 1989). In this respect, LPA acts differently from mitogenic peptides such as serotonin or vasopressin which fail to stimulate cell pro-

liferation unless synergizing growth factors are present (Rozengurt 1986; Moolenaar 1991).

3.1 Structure-Activity Relationship

The effect of LPA and PA on cell proliferation appears to be highly specific. All common lipids, other than PA and LPA, are incapable of stimulating DNA synthesis in quiescent fibroblasts (Moolenaar et al. 1986; Van Corven et al. 1989). The relative potencies of various LPA and PA analogs on fibroblast proliferation have recently been determined (Van Corven et al. 1992). Among the LPAs, $C_{18:1}$-LPA (1-oleoyl) and $C_{16:0}$-LPA (1-palmitoyl) show the highest activity, while the potency decreases when the fatty acid chain length is decreased. In fact, the short-chain LPA, 1-decanoyl LPA, shows hardly any mitogenic activity on quiescent fibroblasts. A similar order of activity is observed with PA as a stimulus (1,2-dioleoyl PA = 1,2-dipalmitoyl PA $\gg$ 1,2-dimyristoyl PA $\gg$ 1,2-dilauroyl PA). An ether-linked LPA (1-*O*-hexadecyl-glycerol-3-phosphate) has strongly reduced mitogenic activity when compared to the ester-linked analog at concentrations $< 25\ \mu M$, and becomes cytotoxic at higher concentrations. Hexadecylphosphate, which lacks a glycerol backbone, has negligible activity. The finding that long-chain LPA and PA are much more active than the short-chain analogs is consistent with a model in which the degree of partitioning into the lipid bilayer is a major determinant of mitogenic potency of these phospholipids.

3.2 Suramin as an Antagonist

In a search for potential antagonists we first focussed on (L)PA analogs possessing the weakest mitogenic activity, i.e., the decanoyl derivatives. Although dilauroyl-PA has been reported to antagonize both short- and long-term effects of long-chain diacyl-PAs (Krabak and Hui 1991; Murayama and Ui 1987), in our hands the short-chain (L)PA analogs lack significant antagonist activity when tested in mitogenic assays. The polysulfonated compound suramin, however, was found to inhibit both LPA and PA-induced DNA sythesis in a reversible and dose-dependent manner ($IC_{50} \approx 70\ \mu M$; Van Corven et al. 1992). The drug appears to act at an early point in the prereplicative phase of the cell cycle and inhibits early biochemical responses to LPA, such as phosphoinositide hydrolysis; importantly, suramin does not interfere with cell proliferation and signal transduction in general, as evidenced by results obtained with EGF and

endothelin, respectively. Suramin has previously been reported to inhibit growth factor-receptor interactions, particularly those of PDGF, transforming growth factor-β and insulin-like growth factor-1 (Hosang 1985; Betsholtz et al. 1986; Coffey et al. 1987; Pollak and Richard 1990). Suramin does not cross cell membranes (Fortes et al. 1973) and, although the drug may be taken up by endocytosis, our results support the view that suramin acts extracellularly rather than intracellularly. Suramin can form complexes with proteins, thereby exerting profound effects on protein tertiary structure, which might explain its ligand displacing properties (Müller and Wolpert 1976). In view of these considerations, our findings obtained with suramin would agree with the hypothesis that (L)PA action is mediated by a specific cell surface receptor. Caution is needed, however, since polyanionic compounds such as suramin may exert their effects by nonspecifically binding to the cell surface; furthermore, we cannot rule out that suramin may interact with the (L)PA molecule itself, rendering it biologically inactive, although reports on complex formation between suramin and phospholipids are not known to us.

The "receptor hypothesis" is, of course, not necessarily incompatible with a "bilayer partitioning" model to explain (L)PA action. Inserted long-chain (L)PA might diffuse laterally within the plane of the lipid bilayer and then have access to a putative receptor which could recognize the glycerolphosphate moiety or the phosphate head group.

4 Mechanism of Action

4.1 Signal Transduction Pathways

At least two types of mitogenic signaling mechanisms can be distinguished, which are used by different classes of cell surface receptors. Receptors for peptide growth factors such as EGF and PDGF are transmembrane proteins with a cytoplasmic domain that functions as a tyrosine-specific protein kinase. Ligand binding causes an immediate stimulation of the catalytic domain, resulting in receptor autophosphorylation as well as in the phosphorylation of various cellular substrate proteins (Ullrich and Schlessinger 1990; Carpenter and Cohen 1990).

G-protein-linked receptors are members of the superfamily of seven transmembrane domain receptors and communicate indirectly to membrane-bound effector enzymes that, upon activation, generate various intracellular messenger molecules. The interaction between the receptor and the effector is mediated by guanosine triphosphate (GTP)-binding

regulatory proteins (G proteins). Adrenergic receptors, receptors for neuropeptides, some of which are mitogenic, and also the receptors for phospholipid agonists such as platelet-activating factor and prostaglandins all belong to the class of G-protein-linked receptors.

PA and LPA are potent and reversible mitogens showing similar dose-response relationships (Van Corven et al. 1992). It therefore seems plausible to assume that both lipids act through the same signaling cascades, although formal proof for this notion is lacking at present. LPA and PA mitogenicity is selectively blocked by pertussis toxin (half-maximal inhibition at 0.2 ng/ml), whereas the mitogenic response to EGF remains unaffected (Van Corven et al. 1990); this bacterial toxin is known to ADP-ribosylate and thereby inactivate the G_i/G_o family of GTP-binding proteins (where G_i is the inhibitory G protein of adenylate cyclase). Three separate signal transduction pathways in the action of LPA have been identified. These are: activation of PLC, release of arachidonic acid presumably as a result of phospholipase A_2 activation, and inhibition of adenylate cyclase as will be discussed below. Recent studies by R. van der Bend indicate that LPA also stimulates PLD activity in quiescent fibroblasts (manuscript in preparation). Furthermore, studies on neuronal cells in culture have revealed that LPA causes rapid changes in cytoskeletal organization, a process that is not attributable to previously identified second messenger cascades, as will be discussed below.

Little is known about the metabolic conversions of LPA and PA, when added exogenously to cells in culture; the major products formed are probably the corresponding monoacylglycerols and DGs (Pagano and Longmuir 1985; R. van der Bend, unpublished observations). Mechanistic studies have largely focussed on the mode of action of LPA, being the most potent and structurally simplest lipid mitogen. In a recent report, Jalink et al. (1990) have presented evidence suggesting that previously reported effects of PA on PLC activity are attributable to contamination with lyso-derivatives. In contrast, as mentioned above, the long-term mitogenic effect of PA cannot simply be explained by LPA contamination (Van Corven et al. 1992).

4.1.1 Activation of PLC

Addition of LPA to various cell types evokes an immediate breakdown of inositol phospholipids as measured by the formation of IP_3 and DG (Van Corven et al. 1989; Jalink et al. 1990). LPA-induced activation of PLC results in a rapid but transient rise in Ca^{2+}, which is primarily caused by the release of intracellularly stored Ca^{2+}. Concomitantly, PKC is activated as shown by the phosphorylation of an endogenous 80-kDa protein

substrate (Van Corven et al. 1989). The kinetics and shape of the LPA-induced Ca^{2+} transient are virtually indistinguishable from those elicited by bradykinin and other hormones. Furthermore, LPA-induced Ca^{+2} mobilization is subject to homologous desensitization, a common feature of many signaling systems where agonist-induced attenuation of cellular responsiveness is thought to have an important regulatory role (Benovic et al. 1988).

From studies on permeabilized cells it appears that the response is GTP-dependent, suggesting the involvement of a G protein (that is insensitive to pertussis toxin in human fibroblasts; Van Corven et al. 1989). Thus, the early phosphoinositide-breakdown response to LPA has many of the hallmarks of a receptor-mediated event. Yet, the exact mechanism by which externally added LPA activates GTP-dependent phosphoinositide hydrolysis is not yet clear. Using La^{3+} as a cell-impermeant "LPA chelator", Jalink et al. (1990) suggested that LPA exerts its immediate effects via a reversible interaction with an external site on the cell surface. The results obtained with suramin, mentioned above, are in agreement with this notion. These findings, together with the rapidily of the onset of the Ca^{2+} signal, argue against a mechanism that requires transbilayer "flip-flop" or internalization of the lipid, although later responses, particularly DNA synthesis, may indeed involve this action.

4.1.2 Release of Arachidonic Acid

LPA, in common with many Ca^{2+}-mobilizing agonists, promotes production of free arachidonic acid, the polyunsaturated fatty acid that is the precursor of prostaglandins and other lipid messengers. Arachidonate can be released from lipids through activation of phospholipase A_2 as a consequence of increased cytoplasmic Ca^{2+} levels following PLC activation. Treatment of the cells with phorbol ester completely blocks LPA-induced phosphoinositide hydrolysis, without significantly affecting arachidonate liberation. Thus, phorbol ester treatment dissociates LPA-mediated arachidonate release from phosphoinositide breakdown. Studies on permeabilized cells suggest that arachidonate liberation by LPA occurs in a GTP-dependent manner, insensitive to pertussis toxin. The precise biochemical mechanisms underlying this early response to LPA remains to be investigated.

4.1.3 Inhibition of Adenylate Cyclase

Both LPA and PA have been reported to inhibit cAMP accumulation in intact cells (Proll et al. 1985; Murayama and Ui 1987; Van Corven et al. 1989). When adenylate cyclase is pre-stimulated by either forskolin or cer-

tain receptor agonists (isoproterenol, prostaglandin E_1, etc.), addition of LPA decreases cAMP accumulation by 60%–70% within 10 min. This decrease in cAMP is dose-dependent and is completely blocked by pretreating the cells with pertussis toxin, indicating that LPA acts through the G_i protein that inhibits adenylate cyclase.

4.1.4 Changes in Neuronal Cell Shape: A Novel Signaling Mechanism

In a recent series of experiments, we have used the neuronal cell lines N1E-115 and NG108-15 as a model system to explore the neurobiological actions of LPA. These cells constitute a convenient model system to investigate early signaling events in the proliferative response. After serum starvation, these cells stop growing and subsequently they begin to acquire various differentiated properties of mature neurons, including the formation of long neurites. Addition of 1-oleoyl-LPA (1 μ*M*) to growth factor-starved N1E-115 or NG108-15 cells causes rapid and dramatic changes in cell shape reminiscent of those observed during mitosis. Virtually every flattened cell starts to round up as early as 5–10 s after LPA addition, while rounding is complete within approx. 1 min. Almost simultaneously, growth cones begin to collapse and developing neurites retract. The effects of LPA on neuronal cell shape are dose-dependent, with some rounding detectable at doses as low as 10 n*M* and maximal responses at 0.5–1 μ*M*. In the continuous presence of LPA, cells maintain their rounded shape for 5–10 min. At 10–20 min after the addition of LPA, however, most cells gradually resume a flattened morphology, but neurite outgrowth remains suppressed for at least a few hours. Remarkably, a second application of LPA to such respread cells leaves the shape unaltered. LPA-induced cell rounding is thus subject to homologous desensitization.

We have established that LPA-induced changes in neuronal cell shape are mediated by cortical actin filaments with no direct involvement of microtubules.

4.1.5 Site of Action and Signal Transduction Pathways

To examine at which cellular site LPA initiates its action, we microinjected LPA into individual N1E-115 cells and scored them for morphological changes. Even when microinjected at high concentrations (100 μ*M*), LPA failed to alter cell shape. However, when LPA-microinjected cells were subsequently exposed to exogenous LPA, the usual morphological response ensued. Thus, LPA appears to induce cell rounding via an extracellular site of action, supporting the view that LPA functions as an extrinsic agonist rather than an intracellular second messenger.

What signaling mechanism is responsible for this novel biological effect of LPA? In cells as diverse as fibroblasts, platelets, and *Xenopus laevis* oocytes, the first detectable response to LPA is GTP-dependent phosphoinositide hydrolysis with subsequent Ca^{2+} mobilization and activation of PKC. In N1E-115 cells, too, LPA stimulates phosphoinositide hydrolysis leading to rapid Ca^{2+} mobilization. Yet, the following findings indicate that the PLC-Ca^{2+}-PKC pathway is not responsible for the observed changes in cell shape: (1) phosphoinositide-hydrolyzing neurotransmitters, Ca^{2+} ionophores, and PKC-activating phorbol ester all fail to mimic LPA in inducing cell rounding; (2) LPA-induced shape changes are not prevented by downregulating PKC by long-term treatment of the cells with phorbol ester; and (3) cells depleted of internal Ca^{2+} (by addition of ionophore in Ca^{2+}-free medium) show a normal morphological response to LPA despite the absence of a Ca^{2+} transient. Similarly, bacterial toxin-sensitive G proteins, adenylate cyclase, or cyclic nucleotides have no apparent role in mediating LPA-induced shape changes, since LPA action is neither inhibited nor mimicked by treatment of the cells with pertussis or cholera toxin, cAMP and cGMP analogs, or forskolin (10 μM). Finally, the involvement of phospholipase A_2 can be ruled out because potent inducers of this enzyme, such as bradykinin and ionomycin, free arachidonic acid, and prostaglandins have no effect on cell shape.

Taken together, these results suggest that LPA-induced cell rounding is not mediated by known G-protein-linked second messenger cascades. By analogy with the cell rounding induced by active protein tyrosine kinases (Chinkers et al. 1981; Jove and Hanafusa 1987) and microinjected *cdc*2 kinase (Lamb et al. 1990), it seems plausible to assume that specific phosphorylations/dephosphorylations of certain actin-binding proteins (Pollard and Cooper 1986; Stossel 1989) may be responsible for the morphological effects of LPA. In support of this, we observed that LPA-induced cell rounding is blocked by both microinjected vanadate and exogenously added pervanadate, which is a membrane-permeable form of vanadate (Fantus et al. 1989), a widely-used inhibitor of protein tyrosine phosphatases. Furthermore, brief preincubation with such (nonspecific) protein kinase inhibitors as genistein (50 μM), quercetin (50 μM), and staurosporine (1 μM) similarly inhibits cell rounding. (These inhibitors are nonspecific since they act at the ATP-binding site on the catalytic domain of numerous protein kinases with differential dose sensitivities; Akiyama et al. 1987; Rüegg and Burgess 1989). Direct biochemical demonstration of the presumed kinase/phosphatase reactions by which LPA may exert its morphological effect awaits further studies.

4.2 Importance of the Early Signals for Cell Proliferation

A central problem in growth factor signal transduction studies concerns the question as to which, if any, of the early biochemical responses accounts for the late mitogenic effects. Quite unexpectedly, and contrary to common belief (Whitman and Cantley 1988), it seems unlikely that activation of the PLC-Ca^{2+}-PKC pathway is of major importance for mitogenesis in our cells. When phosphoinositide hydrolysis is blocked by phorbol ester there is no effect on LPA-induced DNA synthesis. Furthermore, when PKC activity is functionally removed by long-term treatment with tetradecanoyl phorbolacetate (TPA), LPA is still fully mitogenic (Van Corven et al. 1989). It thus appears that LPA does not rely on the phosphoinositide hydrolysis-PKC cascade to stimulate cell proliferation. Furthermore, bradykinin mimics LPA in stimulating phosphoinositide hydrolysis and arachidonic acid release, at least qualitatively, but fails to elicit a detectable mitogenic response (Van Corven et al. 1989). The latter finding further argues against the view that activation of the PLC cascade is sufficient for mitogenesis, at least in quiescent fibroblasts.

It is also unlikely that LPA-stimulated arachidonic acid release is important for the mitogenic action of LPA. First, addition of exogenous arachidonic acid or prostaglandins to quiescent cells does not stimulate DNA synthesis. Second, the estimated EC_{50} of LPA for arachidonic acid release is at least an order of magnitude greater than for mitogenesis. In this regard, low concentrations of LPA can elicit a significant proliferative response while arachidonic acid release is barely detectable (Van Corven et al. 1989).

The results obtained with pertussis toxin strongly suggest that G_i is critical for LPA-induced mitogenesis. This notion is further supported by the finding that the nonmitogenic peptide bradykinin, which activates G_p (putative G protein activating PLC) but not G_i, fails to simulate DNA synthesis. Interestingly, Seuwen et al. (1988) have inferred a similar important role for G_i from their studies on serotonin-induced mitogenesis.

A novel clue to the mode of action of LPA may come from the observations made on the morphology of differentiating neuronal cells. The acute reorganization of the actin cytoskeleton induced by LPA has not yet been explained at the molecular level, but does not appear to involve known G protein-coupled effectors, notably phospholipases and adenylate cyclase. Instead, pharmacologic characterization of LPA-induced cell rounding suggests that vanadate-sensitive phosphatase/kinase cascades are involved; we have begun to test this hypothesis by monitoring the activity of the *src* protein tyrosine kinase in LPA-treated N1E-115 cells (T. Eichholtz, work in progress). Figure 2 summarizes the various biochemical pathways in the

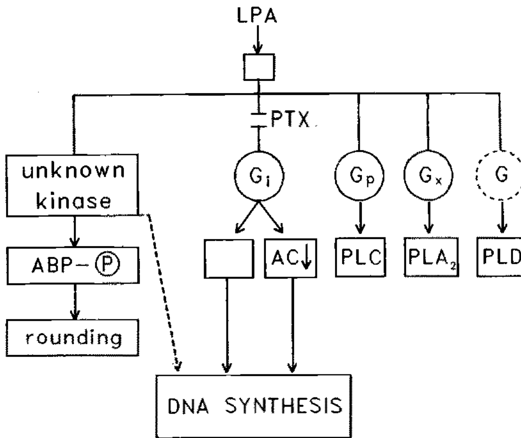

Fig. 2. Proposed scheme of early signal transduction pathways in the action of LPA. *PTX*, pertussis toxin; *AC*, adenylate cyclase; G_x, hypothetical G protein activating PLA_2; *ABP*, actin-binding proteins. The *arrows* denote the direction of cause to effect

action of LPA that have been identified, including the unidentified kinase proposed to act, either directly or indirectly, on certain actin-binding proteins. An unexplained finding is that LPA is about 100 fold more potent in evoking its immediate effects than in inducing DNA synthesis (Jalink et al. 1990).

How could activation of the pertussis toxin-sensitive G_i pathway with subsequent inhibition of adenylate cyclase be important for DNA synthesis? cAMP has long been known as a modulator of cell growth in many cell systems (Rozengurt 1986). In normal fibroblasts, including Rat-1 and human fibroblast (HF) cells, agents that raise intracellular cAMP levels exert a marked growth-inhibitory effect (Heldin et al. 1989). Conversely, it seems reasonable to assume that a reduction in cellular cAMP may have a growth-promoting effect in these cells. Alternatively, G_i or a related member of the pertussis toxin-sensitive G_i family could interact with an as yet unidentified effector system that is important for mitogenesis. This effector system is, however, not identical to the proposed "unknown" kinase activity acting on the cytoskeleton (Fig. 2), since LPA-induced morphological changes are insensitive to pertussis toxin.

4.3 Site of Action

A key question concerns the mechanism by which extracellular LPA activates certain G proteins at the inner side of the plasma membrane. From

microinjection studies and the pharmacologic experiments using suramin and La^{3+} mentioned earlier, it appears that LPA exerts its early effects through an extracellular site of action, possibly a receptor. Membrane-inserted long-chain LPA might diffuse laterally, thereby triggering a G protein-coupled "receptor" which could recognize the phosphate head group. Short-chain LPA (1-decanoyl LPA) might not translocate to the proper site on the plasma membrane, thus being incapable of activating the putative receptor. The finding that responsiveness to LPA is cell-type specific (see Jalink et al. 1990) supports the receptor hypothesis. Another possibility is that inserted LPA perturbs the structure of the lipid bilayer in such a way that G proteins are selectively activated in a receptor-independent fashion. To date, however, there are no data to indicate how LPA might alter bilayer structure; it is equally unclear to what extent the structure and composition of the lipid bilayer may affect G protein function.

It should be mentioned that the mechanism by which LPA exerts its immediate cellular effects at the level of the plasma membrane may well be unrelated to the later responses such as DNA synthesis and cell proliferation. At present it cannot be ruled out that transbilayer flip-flop or internalization of the lipid with subsequent metabolic conversion is required for LPA-induced mitogenesis. Clearly the importance of metabolic conversion of exogenous LPA for long-term biological responses remains to be addressed.

5 Other Biological Effects

As LPA is a potent activator of certain G protein-coupled effector systems, it may serve as a convenient probe to identify and dissect various biological responses in different cell systems. Indeed, LPA has been used to monitor cellular effects and to activate second messenger pathways in various cell types. The results of these studies are summarized in Table 1. It is seen that cells as diverse as mammalian fibroblasts, platelets, and *Xenopus laevis* oocytes share a common rapid response to LPA, i.e., activation of the G protein-mediated PLC-second messenger cascade. Yet, responsiveness to LPA is not a universal characteristic of all cell types: human neutrophils and monocytes fail to show a detectable rise in cytoplasmic [Ca^{2+}], even when challenged with high concentrations of the lipid (Jalink et al. 1990, and unpublished observations). Also, rat mast cells, which rapidly degranulate in response to Ca^{2+}-mobilizing agents, fail to do so when stimulated with LPA (B. Gomperts, personal communication). It thus seems as if cells of the immune system are nonresponsive to LPA. Other leukocytes should

Table 1. Early cellular responses to LPA

Type of Cell	Responses
Fibroblasts	Ca^{2+} mobilization; arachidonate release; inhibition of adenylate cyclase
A431 epidermoid carcinoma cells	Ca^{2+} mobilization
Chicken osteoblasts	Ca^{2+} mobilization[a]
COS (kidney) cells	Ca^{2} mobilization[a]
Neuroblastoma cells	Ca^{2+} mobilization; morphological changes[a]
HT-29 colon carcinoma cells	Cl^{-} secretion[b]
MDCK epithelial cells	Cl^{-} secretion[b]
Mouse embryonic stem cells	Ca^{2+} mobilization[c]
Smooth muscle	Ca^{2+} mobilization; contraction
Platelets	Ca^{2+} mobilization; aggregation[d]
Xenopus laevis oocytes	Ca_i^{2+}-dependent Cl^{-} current[c]
Dictyostelium disc	chemotaxis[e]

References can be found in the text unless indicated otherwise.
[a] K. Jalink, unpublished observations. [b] H. de Jonge, unpublished observations. [c] L. Tertoolen, unpublished observations. [d] Tokumura et al. (1980); see also Salmon and Honeyman (1980). [e] B. van Duin, personal communication.

be examined for their LPA responsiveness before general statements can be made. Nevertheless, the finding that all lymphoid cells that have been tested lack a detectable response to LPA is intriguing and may contribute to identifying the nature of the putative LPA receptor or the specific intracellular targets.

6 Possible Biological Function and Future Prospects

The discovery that LPAs exert profound effects on many, but not all, cell types suggests that these low-abundance lipids may have a physiological role as extrinsic lipid mediator. An interesting possibility is that LPA (which is fairly well water-soluble) is secreted by cells in a manner similar to the secretion of platelet-activating factor, prostaglandins, and other lipid agonists. Indeed, preliminary experiments suggest that LPA is secreted in a biologically active form by certain leukocytes (T. Eichholtz and K. Jalink, unpublished observations). The released LPA may then activate target cells in a paracrine or autocrine fashion. Phospholipid labeling studies will allow a critical test of this hypothesis. Finally, several reports have described a phospholipase D in mammalian plasma that is specific for glycosylphosphatidylinositol (GPI) serving as a membrane anchor for

surface proteins (Davitz et al. 1989; Low and Prasad 1988). This GPI-specific PLD may function not only to liberate the anchored protein from the cell surface, but also to produce biologically active PA in the outer leaflet of the plasma membrane. The precise physiological role of GPI-specific PLD activity remains to be determined.

In summary, long-chain LPAs are potent mitogens for various mammalian cell types and recent evidence suggests that LPA acts, at least in part, by activating a subset of the family of membrane-bound G proteins including G_i and G_p. It is likely, however, that as yet unidentified effectors are involved in LPA action: LPA appears to activate effector enzymes acting on the neuronal cytoskeleton in a G protein-independent manner (Fig. 2).

Whether or not LPA binds to and thereby activates a cell-type specific cell surface receptor awaits further investigations. Another unanswered question concerns the finding that LPA is about two orders of magnitude more effective in activating PLC and inhibiting adenylate cyclase than in inducing DNA synthesis (Jalink et al. 1990; Van Corven et al. 1991). It is conceivable that higher concentrations of LPA are required for such long-term effects as DNA synthesis due to cellular uptake and/or metabolic conversion of LPA. An alternative, but not mutually exclusive, possibility is that the early signaling events and the ultimate mitogenic response are causally unrelated in that they might be generated by different mechanisms with distinctly different LPA requirements. Future experiments, designed to evaluate the metabolic fate and the cellular targets of LPA, should help in resolving the discrepancy between the dose-response curves. Such experiments should also clarify to what extent the report effects of PA are attributable to contamination with LPA.

Whatever the precise site of action and biological function of LPA may be, this simple phospholipid may serve as a potent pharmacologic tool to dissect the role of G-protein-mediated signaling pathways, particularly those involved in growth control. The design and synthesis of biologically inactive LPA analogs that inhibit LPA action, and of fluorescent and photoactive analogs could greatly contribute to the identification of the cellular receptor(s) for LPA.

Acknowledgements. The secretarial assistance of Paulien Sobels is gratefully acknowledged. Research related to this review was supported by the Netherlands Cancer Foundation and by the Netherlands Organization for Scientific Research (NWO).

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Rev. Physiol. Biochem. Pharmacol., Vol. 119
© Springer-Verlag 1992

Calcium Signals in Growth Factor Signal Transduction

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1 Introduction

The regulation of cytosolic free calcium concentration ($[Ca^{2+}]_i$) is of crucial importance to cell function. In fact, the abilities of cells to maintain $[Ca^{2+}]_i$ within a very restricted range and to respond to extracellular stimuli with tightly controlled changes in $[Ca^{2+}]_i$ are central to nearly every aspect of our physiology. From the fertilization of an egg to the control of cell growth, the beating of our hearts, transmission of nervous impulses, contraction of skeletal and vascular muscle, clotting of blood, secretion of hormones and digestive enzymes – all are directly regulated by cytosolic Ca^{2+}.

This tight regulation of cytosolic $[Ca^{2+}]$ is fundamental to the Ca^{2+} signal transduction process whereby an extracellular stimulus is transduced into a cellular response which involves some change in $[Ca^{2+}]_i$. While physiological stimuli and the types of responses they elicit vary greatly among different cell types in different tissues, the cellular machinery for regulating $[Ca^{2+}]_i$ is highly conserved. In this review article we will discuss the various aspects of regulation of intracellular Ca^{2+}, how $[Ca^{2+}]_i$ is measured and how $[Ca^{2+}]_i$ can be modified by stimulating proliferating

cells with growth factors. Although the main focus of this review is the Ca^{2+} response in proliferating cells, much of the important data discussed will have been collected in nonproliferating cells. In some cases, data in nonproliferating cells provides the crucial evidence for the existence of a particular phenomena that we will go on to discuss in proliferating cells, where there may not be as much in-depth evidence as exists in nonproliferating cells.

2 Regulation of Intracellular Ca^{2+}

Prior to a discussion of the role of intracellular Ca^{2+} in the growth factor signal transduction process, it is important to consider briefly (we will discuss the processes in more depth later in this review) the mechanisms involved in maintaining the basal Ca^{2+} level and how these mechanisms might be modified to allow a dramatic rise in $[Ca^{2+}]_i$. Cells regulate $[Ca^{2+}]_i$ very closely, maintaining basal $[Ca^{2+}]_i$ in the range of 50–200 n*M*, while extracellular Ca^{2+} is normally at millimolar concentrations. This is accomplished by extrusion of Ca^{2+} across the plasma membrane and by sequestration of Ca^{2+} within the cell either by pumping Ca^{2+} into intracellular compartments or by binding of Ca^{2+} to cell structures and cytosolic proteins. Extrusion of Ca^{2+} from the cell is performed primarily by two components of the plasma membrane. A Ca^{2+}-ATPase, fueled by the hydrolysis of ATP, pumps Ca^{2+} out of the cell (Carafoli 1987; Carafoli et al. 1989, 1990; Caroni et al. 1981; Dean 1989; Burgoyne et al. 1989; Furukawa et al. 1989; Inesi and Kirtley 1990) and a Na^+/Ca^{2+} exchanger removes intracellular Ca^{2+} at the expense of a Na^+ gradient across the plasma membrane (Sheu and Blaustein 1986; Lagnado and McNaughton 1990). These extrusion mechanisms serve to offset the continued leak flux of Ca^{2+} into the cytoplasm from the extracellular space. The leak of Ca^{2+} occurs through Ca^{2+} channels present in the plasma membrane. There also exist intracellular compartments which sequester Ca^{2+} via Ca^{2+}-pumping mechanisms. These internal compartments include the endoplasmic reticulum and mitochondria (Gunter and Pfeiffer 1990). Specialized Ca^{2+}-sequestering organelles exist which are cell type specific. These include the sarcoplasmic reticulum of muscle fibers (James et al. 1989) and the dense tubular system of platelets. The membranes of the intracellular organelles can contain Ca^{2+} channels which in some cases can be controlled by internal signalling molecules [(e.g., $Ins(1,4,5)P_3$] to cause release of Ca^{2+} into the cytoplasm. Later in the manuscript we will discuss the pump and leak systems in more depth and

then discuss the effects of growth factors on the various parameters which control cytosolic Ca^{2+} concentration.

3 Early Evidence for Ca^{2+} Involvement in Growth Factor Signal Transduction

One very important physiological process for which the Ca^{2+} signal transduction process has been implicated is the control of cell growth. Early evidence for Ca^{2+} involvement in cell growth control came from a number of indirect measurements of Ca^{2+} involvement, since this was during a time in which, due to technical limitations, the free intracellular Ca^{2+} concentration could not be readily measured in most cell types. Instead, investigators depended on experiments whereby Ca^{2+} was removed from cells and their growth rates measured; intracellular Ca^{2+} was artificially elevated with Ca^{2+} ionophores to determine the effect on cell growth, or the movements of Ca^{2+} across cell membranes were measured in response to growth factor stimulation to reflect the importance of Ca^{2+} in regulating cell growth.

The earliest postulation of Ca^{2+} involvement in cell growth was put forward by Mazia in 1937. He used very crude methods to try to access the level of unbound Ca^{2+} in *Arbacia punctulata* eggs to determine whether this parameter changed with fertilization of the egg. By this crude method he suggested that the level of free Ca^{2+} did increase with fertilization but his values were orders of magnitude above what we consider today to be the true free Ca^{2+} concentration.

Further support for the involvement of Ca^{2+} in regulation of cell growth would not come for many years. Due to the above mentioned limitations in measuring $[Ca^{2+}]_i$, experimental support for a role of Ca^{2+} in regulation of cell growth came in the form of data showing that artificially elevating $[Ca^{2+}]_i$ could activate certain cell types, that stimulation of cells with growth factors would increase Ca^{2+} fluxes in cells, and that removing external Ca^{2+} could block the activation of cell growth by mitogenic agents.

Initial evidence for Ca^{2+} involvement came from Ca^{2+} replacement studies in lymphocytes and in fibroblasts. Several early studies (Alford 1970; Whitney and Sutherland 1972) demonstrated the central importance of Ca^{2+} in the growth of lymphocytes. It was observed that external Ca^{2+} was necessary for the phytohemagglutinin (PHA) induced lymphocyte transformation response. The morphological transformation as well as DNA synthesis induced by PHA could be inhibited by the addition of

ethylenediamine tetra-acetic acid (EDTA) or ethylene glycol tetra-acetic acid (EGTA) to the external medium. This blockage could be reversed by the addition of excess Ca^{2+}.

Ca^{2+} removal studies were also reported in fibroblasts that were stimulated to divide with serum. Several reports (Balk et al. 1973; Boynton et al. 1977; Hazelton et al. 1979) demonstrated that fibroblasts would arrest if the external Ca^{2+} concentration was reduced from normal physiological levels ($1-2\,mM$) to much lower levels ($10-100\,\mu M$). Complex experiments were performed to determine where in the cell cycle the arrest occurred in Ca^{2+}-free medium. In a human lung fibroblast (WI-38 cells) Boynton et al. (1977) demonstrated that DNA synthesis was greatly reduced for cells incubated for several days in fetal bovine serum-Eagle's basal medium (FBS-BME) containing approximately $100\,\mu M$ Ca^{2+}. They observed that readdition of Ca^{2+} to the growth medium ultimately led to the induction of DNA synthesis. The short time period between readdition of Ca^{2+} and the initiation of DNA synthesis indicated that at least a large fraction of the population was arrested late in the G_1 phase of the cycle. Another population of cells began synthesizing DNA 12 h after the readdition of Ca^{2+}, suggesting that at least some of the cells were arrested at an earlier stage of G_1.

An interesting observation coming from the above studies was that while normal fibroblasts are highly dependent on the presence of external Ca^{2+}, transformed fibroblasts can grow quite well in the absence of external Ca^{2+}. Studies in Rous sarcoma virus-transformed chick embryo fibroblasts (Balk et al. 1973) and SV40 virus-transformed human cells (Boynton et al. 1977) indicate that transformed cells grow very well in media with Ca^{2+} levels which would cause a dramatic arrest of normal cells. The basis for the relative Ca^{2+} independence of the transformed cells is still not understood.

While the Ca^{2+} replacement experiments suggested that Ca^{2+} might be necessary for cell proliferation, they did not indicate whether the addition of mitogenic agents to quiescent cells had any effect on intracellular Ca^{2+}. During the time that the Ca^{2+} removal experiments were ongoing, there were also many studies being performed to determine the effect of growth factors on Ca^{2+} transport in proliferating cells. These studies questioned whether growth factors could induce a change in Ca^{2+} transport across cellular membranes, which might be responsible for changes in intracellular Ca^{2+}. At that time it was not possible to measure intracellular free Ca^{2+} concentrations in cells such as fibroblasts and lymphocytes, thus measurement of Ca^{2+} movement via transport pathways (which might influence $[Ca^{2+}]_i$) was the best that could be done. Changes in Ca^{2+} transport with addition of growth factors generally were interpreted

as an indication that growth factors could change the intracellular free Ca^{2+} concentration.

Studies by Allwood et al. (1971) demonstrated a PHA-induced change in $^{45}Ca^{2+}$ influx in lymphocytes. In another study by Freedman et al. (1975), a more rapid change in $^{45}Ca^{2+}$ influx was reported for lymphocytes stimulated by concanavalin A. This stimulation of $^{45}Ca^{2+}$ influx was transient and was observed to be over within 5 min.

Numerous $^{45}Ca^{2+}$ transport studies were performed in fibroblasts during this time period. These included both Ca^{2+} influx (Tupper et al. 1978; Hazelton and Tupper 1979) and Ca^{2+} efflux (Owen and Villereal 1983) measurements in cells stimulated with mitogenic agents. Clearly, investigators saw changes in the rate of influx and efflux of $^{45}Ca^{2+}$ when fibroblasts were stimulated with growth factors. However, the interpretation of the underlying cause of an increase in Ca^{2+} flux in response to mitogenic stimulation was very complicated. Due to the existence of multiple internal compartments in fibroblasts the analysis of either Ca^{2+} influx or Ca^{2+} efflux data is complex (for a discussion of these complexities see Villereal 1991). In general, the large number of flux studies suggested that growth factors were indeed stimulating an influx of Ca^{2+} and probably a mobilization of intracellular Ca^{2+}, but the exact mechanisms involved were not clear.

Thus far, we have considered evidence which indicated that cells need Ca^{2+} to grow and that growth factors cause a change in $^{45}Ca^{2+}$ transport, which theoreticially could lead to an elevation of $[Ca^{2+}]_i$. What remained was to show that Ca^{2+} was causative in the induction of cells to proliferate. A number of studies attempted to get at this question by determining whether an artificially induced elevation of $[Ca^{2+}]_i$ could cause a cell to proliferate. Initial success in activation of cells by artifically elevating $[Ca^{2+}]_i$ came in two cell systems, the lymphocyte and the sea urchin egg. These investigations involved the use of Ca^{2+} ionophores such as A23187 to artificially elevate the $[Ca^{2+}]_i$ level in the absence of mitogenic agents to determine whether one would obtain a stimulation of DNA synthesis. Results in sea urchin eggs illustrated that A23187 could induce many of the events seen following fertilization (Steinhardt and Epel 1974). That is, A23187 could induce increases in protein synthesis, respiration, DNA synthesis and a hyperpolarization of the membrane; all of which were similar in nature to those effects seen upon fertilization. Similar results were reported in lymphocytes, where blast transformation and DNA synthesis could be induced by A23187 (Luckasen et al. 1974). Although studies in both sea urchin eggs and lymphocytes were quite successful in showing that an artificial elevation of $[Ca^{2+}]_i$ could induce many features of the proliferative response, similar studies undertaken in fibroblasts were not successful.

4 Measurement of $[Ca^{2+}]_i$ in Growth Factor-Stimulated Cells

The aforementioned evidence, accumulated in the early 1970s, suggested that Ca^{2+} is involved in the regulation of cell growth, but up to that time, no direct measure of the $[Ca^{2+}]_i$ of proliferating cells had been made to demonstrate that growth factors actually do regulate $[Ca^{2+}]_i$ levels. The initial system in which such measurements were made was the Medaka egg (Ridgway et al. 1977), an egg from a fresh water fish which is both very large (1.2 mm in diameter) and transparent. This egg was microinjected with the Ca^{2+}-sensitive, light-emitting protein aequorin. Due to its large size it could be easily microinjected and still remain intact to respond to fertilization. The light emitted from the aequorin was monitored as a measure of the $[Ca^{2+}]_i$. The initial low light level suggested a basal Ca^{2+} of about 100 n*M*. Fertilization of the egg led to a dramatic increase in light output and presumably a dramatic increase in $[Ca^{2+}]_i$. In addition to its use in studies utilizing aequorin to measure $[Ca^{2+}]_i$, these eggs (and sea urchin eggs) were utilized for other microinjection experiments. For example, the Ca^{2+} chelator EGTA was microinjected into the eggs and found to block the cortical vesicle discharge and formation of the fertilization membrane (Zucker and Steinhardt 1978).

4.1 Ca^{2+}-Sensitive Fluorescent Dyes for Ca^{2+} Measurement in Small Proliferating Cells

Although the microinjection of aequorin allowed investigators to monitor $[Ca^{2+}]_i$ in large egg systems, this technique could not be adopted to smaller proliferating cells such as fibroblasts and lymphocytes. However, in 1980 Roger Tsien introduced a new Ca^{2+} probe, quin2, which could be loaded into smaller proliferating cells and would enable investigators to monitor intracellular Ca^{2+} levels in response to growth factors (R. Y. Tsien 1980, 1981; R.Y. Tsien et al. 1982a).

Quin2 was developed to approximate the structure of EGTA, a molecule which was known to have a high selectivity for Ca^{2+} binding. The four carboxyl group structure which comprises the Ca^{2+} binding site of EGTA was incorporated into a ring structure which provided the fluorescent signal necessary to monitor Ca^{2+} binding. This provided a probe whose fluorescence output was very sensitive to the level of Ca^{2+}. Since quin2 had a K_d of 115 nm, it could serve as a valuable tool for monitoring $[Ca^{2+}]_i$. However, before it could be widely used, a convenient method for loading quin2 into small cells had to be achieved. This was accomplished by masking the four carboxyl groups with ester groups to make the

modified form of quin2 lipophilic so that it could easily permeate the plasma membrane. Thus, the acetoxy methyl ester of quin2 could readily cross the plasma membrane and enter even very small cells. Once inside the cell, the probe would encounter cytosolic esterases which would remove the ester group, thereby generating the Ca^{2+}-sensitive form of the fluorescent probe. A number of controls ensured that the quin2 loaded principally into the cytoplasm where it could accurately reflect changes in cytosolic Ca^{2+} concentration (Tsien et al. 1982a).

The initial use of quin2 was to monitor changes in $[Ca^{2+}]_i$ in lymphocytes (R.Y. Tsien et al. 1982b) to determine whether addition of mitogenic agents would modify $[Ca^{2+}]_i$, as had been predicted for several decades based upon Ca^{2+} flux studies. Lymphocytes were loaded with quin2 and then placed in a cuvette in a fluorometer and excited with light of wavelength 339 nm and the fluorescence output continuously monitored. It was reported that addition of concanavalin A to lymphocytes resulted in a significant rise in $[Ca^{2+}]_i$. Once quin2 became commercially available, a number of studies soon followed utilizing quin2 to monitor intracellular Ca^{2+} in a variety of other suspension cells (Berthon et al. 1984; Hallam et al. 1984).

Although the cuvette-based fluorescence measurements were useful for studies in suspension cells such as lymphocytes and platelets a modification of these techniques had to be made in order to measure intracellular Ca^{2+} in attached cells such as fibroblasts. Several groups (Rogers et al. 1983; Mix et al. 1984; Moolenaar et al. 1984b) adapted the quin2 techniques to the measurement of $[Ca^{2+}]_i$ in fibroblasts by monitoring the fluorescence of cells grown on a coverslip. The cells were grown on a microscope slide and the fluorescence monitored either by placing the slide in a cuvette of a fluorometer or by recording the signal from a microscopic field of cells monitored with a photon counter to quantitate the signal from the cells. These studies demonstrated that the addition of serum and purified growth factors to quiescent cells would result in a significant increase in the cytosolic Ca^{2+} concentration of fibroblasts.

4.2 New Generation of Ca^{2+}-Sensitive Fluorescence Indicators

Although the design and synthesis of quin2 represented a major breakthrough in the measurement of intracellular Ca^{2+} in small cells, there were some definite shortcomings to the use of quin2 as a probe for intracellular Ca^{2+}. The quantum yield of quin2 was low; consequently a large amount of the dye (around 1 m*M*) had to be loaded into the cell in order to obtain a signal. Since quin2 has a high affinity for Ca^{2+}, it can

serve as a Ca^{2+} buffer inside the cell, much as EGTA would. Thus, there were concerns that the values for the transient changes in $[Ca^{2+}]_i$ were being severely underestimated by quin2. This and other potential problems were rectified by the introduction of a new Ca^{2+}-sensitive fluorescence probe called fura2 (Grynkiewicz et al. 1985). Fura2 has a much higher quantum yield than does quin2, thereby necessitating loading much lower quantities of dye into the cell to obtain a measurable signal. Another major improvement in the way Ca^{2+} could be measured was the fact that fura2 changed its spectrum when it bound Ca^{2+}. When excited at 340 nm, fura2 increases its fluorescence when Ca^{2+} is bound, while when excited at 380 nm fura2 decreases its fluorescence when Ca^{2+} is bound. Thus, by ratioing the fluorescence signals at 340 and 380 excitation, a very accurate indication of the changes in intracellular Ca^{2+} concentration can be obtained.

Calibration of the ratio technique can be obtained by one of two methods. First, an in vitro calibration of fura2 can be performed whereby a series of Ca^{2+}/EGTA buffers of varying free Ca^{2+} concentrations is prepared. The fura2 340:380 ratio at each concentration of Ca^{2+} is then measured, and it is then assumed that the fura2 inside the cell behaves in a similar manner as does the dye in the buffer system. The standard curve generated in vitro is then used to estimate the $[Ca^{2+}]_i$ for a given 340:380 ratio recorded in the cell. However, several recent publications indicate that the dye does not behave in exactly the same manner inside the cell as observed in a buffer system (Poenie 1990; Konishi et al. 1988). To minimize the differences in environment seen in the cell and during the in vitro calibration, it is possible to calibrate the fura2 fluorescence while the probe is still in the cytoplasm of the cell. After an experiment, one adds ionomycin to allow the external Ca^{2+} to equilibrate with the internal compartment and thereby elevate the Ca^{2+} to a saturating Ca^{2+} concentration. One can then add EGTA to chelate the external Ca^{2+} and, assuming the cytoplasmic Ca^{2+} concentration is in equilibrium with the outside, one can then calculate the fura2 ratio for a Ca^{2+}-free condition. Using the minimum and maximum fluorescence ratios and the K_d for fura2 binding of Ca^{2+}, one can calculate $[Ca^{2+}]_i$ for any measured fura2 ratio. A more detailed description of the technical aspects of working with fura2 is available elsewhere (Villereal 1991).

With the introduction of fura2, measurements of $[Ca^{2+}]_i$ became much more reliable. Data began to appear from a number of different laboratories showing that mitogenic agents could cause an increase in cytosolic Ca^{2+} concentration in fibroblasts. Depending on the mitogen used to stimulate the cells the rise in $[Ca^{2+}]_i$ could be due to release of sequestered Ca^{2+} from intracellular stores, influx of Ca^{2+} across the plasma

membrane, or decreased Ca^{2+} extrusion, or some combination of these events. Evidence that mitogens stimulate release of Ca^{2+} from intracellular pools comes most convincingly from studies which utilize fluorescent Ca^{2+} indicators to continuously monitor $[Ca^{2+}]_i$ following mitogenic stimulation of cells. In many cell systems, mitogenic stimulation leads to a transient elevation of $[Ca^{2+}]_i$ even in the absence of Ca^{2+} in the extracellular medium (Moolenaar et al. 1984b; Palfrey et al. 1987; Tucker et al. 1988). However, in addition to Ca^{2+} mobilization from internal stores agonists also stimulate Ca^{2+} influx. This has been demonstrated by several studies that clearly distinguish influx from decreased efflux (e.g., unidirectional $^{45}Ca^{2+}$ flux experiments [Exton 1987] and studies which monitor Mn^{2+} quenching of fura2 fluorescence [Hallam et al. 1988, 1989; Merritt and Hallam 1989; Jacob 1990]).

4.3 Image Analysis Techniques in the Measurement of Cytosolic Ca^{2+}

Recent developments in computer assistance in the analysis of microscopic images has had a dramatic effect on the way one can investigate the regulation of intracellular Ca^{2+}. In the original studies of $[Ca^{2+}]_i$ in fibroblasts the measurement of Ca^{2+} was with microspectrofluorometers (Rogers et al. 1983; Mix et al. 1984), in which photons are integrated from all the cells and therefore the signal affords no spatial information. Some spatial information could be obtained by closing down the diaphragm on the excitation side of the microscope so that only a single cell is excited and thereby obtaining the Ca^{2+} response of this individual cell. However, stimulation of this cell by growth factors means that all of the cells in the field were simultaneously stimulated. Thus, in order to record a second naive cell, one has to change to a fresh coverslip of cells. One way to avoid the time-consuming task of changing coverslips for each cell recorded would be to record the response of each and every cell in the field simultaneously. This can be done if the photon counter of the microspectrophotometer is replaced with a high sensitivity TV camera, such as a SIT camera or a Nevicon camera in series with an image intensifier. The output of the TV camera is then fed into some type of image analysis system which can digitize the image. Thus, a number value (gray level ranging from 0 to 255) is stored for the level of intensity for each pixel in the image. This information can be stored in the memory of the computer, on a hard drive or on an optical disk for future analysis of the image. During a typical Ca^{2+} experiment one collects both an image at 340 excitation and an image at 380 excitation for each time point of interest. Upon later analysis of the data, the computer divides the gray level values in the 340 image by the gray level values

in the 380 image for each and every pixel. The ratio computed for each pixel in the image is then converted to a Ca^{2+} value for each pixel, based on the standard curve of ratio versus Ca^{2+} concentration. At this point, most Ca^{2+} analysis programs allow the investigator to chose an area in the field and average the Ca^{2+} values over that area. Therefore, a cell, or a region within a cell, is outlined with a light pen and an average Ca^{2+} value over that individual cell is obtained. This process can be repeated for every cell in the field. Since there can be 30–40 cells in a field, individual Ca^{2+} responses can be rapidly obtained for a large number of cells.

4.3.1 Information Obtained from the Application of Image Analysis to Ca^{2+} Measurements in Cultured Cells

The introduction of microfluorometry and image analysis techniques has enabled investigators to monitor changes in $[Ca^{2+}]_i$ in individual cells. The use of these techniques has revealed a surprising degree of heterogeneity of responses in populations of cultured cells. Weniger et al. (1987) examined $[Ca^{2+}]_i$ changes in cultured rat lactotrophs responding to dopamine or thyrotropin-releasing hormone. They reported considerable variability in the magnitude and duration of each of two phases of the $[Ca^{2+}]_i$ responses of individual cells to either of these compounds, even though the cells were all identified as lactotrophs by immunostaining. Monck et al. (1988) observed $[Ca^{2+}]_i$ changes in individual cultured A10 vascular smooth muscle cells and rat hepatocytes stimulated with Arg-vasopressin and phenylephrine, respectively, which were heterogeneous with respect to the shapes of the $[Ca^{2+}]_i$ time courses. They also reported asynchrony of the onsets of $[Ca^{2+}]_i$ changes in individual cells stimulated with submaximal doses of agonist. Millard et al. (1988) also reported asynchronous changes in $[Ca^{2+}]_i$ in tumor mast cells stimulated with antigen. Monitoring $[Ca^{2+}]_i$ in individual cells has also revealed oscillations of $[Ca^{2+}]_i$ which occur spontaneously in some cell types (Holl et al. 1988) or in response to stimulation in others (Prentki et al. 1988; Stuenkel et al. 1989; Berridge et al. 1988). Although a number of laboratories had reported heterogeneous and/or asynchronous $[Ca^{2+}]_i$ responses (Wineger et al. 1987; Monck et al. 1988; Millard et al. 1988; Holl et al. 1988; Prentki et al. 1988; Berridge et al. 1988; Hesketh et al. 1988; Ambler et al. 1988; Stuenkel et al. 1989; Boynton et al. 1989), no experimental evidence had been presented which would serve to elucidate the mechanisms underlying these observations in these various systems.

4.3.2 Asynchrony and Heterogeneity in Ca^{2+} Response in Bradykinin-Stimulated Fibroblasts

Recently, our laboratory has been studying intracellular signals generated by mitogenic stimulation of cultured human foreskin fibroblasts (HSWP cells). We have demonstrated that bradykinin, which is mitogenic in these cells (Owen and Villereal 1983), stimulates a transient increase in $[Ca^{2+}]_i$ in populations of these cells (Muldoon et al. 1987). We have recently reported (Byron and Villereal 1989) that bradykinin produces changes in $[Ca^{2+}]_i$ in individual cells which are both asynchronous and heterogeneous. We investigated the basis for the heterogeneity and asynchrony of the $[Ca^{2+}]_i$ responses. Since cells growing in culture are generally heterogeneous with respect to the cell division cycle, it was important to determine whether asynchrony of individual cell responses was determined by cell cycle differences. We observed that synchronized noncycling cells exhibit the same degree of asynchrony of $[Ca^{2+}]_i$ responses as cycling cells following bradykinin stimulation.

We also sought to determine whether the asynchronous/heterogeneous behavior of cells is a totally random event or if each cell is programmed to respond in a particular reproducible way. We investigated this question by repetitively stimulating HSWP cells with mitogens and observing how subsequent $[Ca^{2+}]_i$ responses compared with the initial response. The perfusion system utilized for these studies allows the rapid exchange of medium bathing the cells. The bradykinin can be washed from the cells, and the same cells can then be exposed for a second time to bradykinin or another mitogen. Repetitive stimulation with bradykinin revealed that each cell in a field responds similarly during successive stimulations. Although some desensitization is apparent, the shape of the $[Ca^{2+}]_i$ time course from a cell which responds to a second exposure to bradykinin is similar to that elicited by the first bradykinin exposure (Byron and Villereal 1989).

Ranking the cells according to the order in which they responded to bradykinin (the first to respond was ranked 1, the second to respond is 2, etc.) revealed that the rank order is maintained quite closely during subsequent stimulations with bradykinin (Byron and Villereal 1989). This data indicated that the responsiveness of an individual cell to bradykinin represents an intrinsic property of that particular cell. This is consistent with the observations of Prentki et al. (1988) that hamster insulinoma cells stimulated with carbamylcholine exhibited characteristic and reproducible $[Ca^{2+}]_i$ responses following each stimulation, which they termed a "Ca^{2+} fingerprint" for each cell. The changes in $[Ca^{2+}]_i$ result from a sequence of biochemical events which are triggered by the mitogen binding to a

specific cell-surface receptor. Evidence from our laboratory (Etscheid 1989; Etscheid and Villereal 1989) suggests that, in HSWP cells, the transduction of bradykinin binding to elevation of $[Ca^{2+}]_i$ involves a G protein coupled to phospholipase C, which cleaves phosphatidylinositol 4,5-bisphosphate into $Ins(1,4,5)P_3$ and diacylglycerol. $Ins(1,4,5)P_3$ is believed to mediate the release of Ca^{2+} from intracellular stores (Berridge and Irvine 1989). This released Ca^{2+} probably produces the initial rise in $[Ca^{2+}]_i$ following exposure to bradykinin. Cell-to-cell variation in any of the components of the signal transduction cascade, from receptor binding to the release of intracellular Ca^{2+}, could account for the heterogeneity observed.

If the heterogeneity of the $[Ca^{2+}]_i$ responses is due to cell-to-cell variation in the number of bradykinin receptors, then one might expect the $[Ca^{2+}]_i$ response to another mitogen to differ from the bradykinin-induced response. (If there is a cell-to-cell variation in receptor number, there is no reason to expect that, given two different mitogens, A and B, cells with a high number of A receptors would also have a high number of B receptors, while cells with few A receptors also have few B receptors.) Since thrombin is a mitogen which produces $[Ca^{2+}]_i$ changes similar to bradykinin and operates through a similar pathway involving PI turnover, we compared the bradykinin and thrombin responses. Experiments in cells stimulated with bradykinin in Ca^{2+}-free medium and then subsequently exposed to thrombin (also in Ca^{2+}-free medium) revealed that the thrombin response was absent, suggesting that the intracellular Ca^{2+} pool that is normally released by thrombin had been depleted by the bradykinin response. This is further evidence that bradykinin and thrombin are acting to release the same intracellular Ca^{2+} stores.

We observed that if a field of cells is exposed to bradykinin and ranked according to the order of response and then washed free of bradykinin and exposed to thrombin, the response order to thrombin does not correspond to the response order following bradykinin stimulation (Byron and Villereal 1989). Furthermore, the shape of the $[Ca^{2+}]_i$ time course of an individual cell following thrombin stimulation does not usually match that following bradykinin stimulation of the same cell. These data suggest that the $[Ca^{2+}]_i$ changes induced by bradykinin or thrombin are characteristic for each mitogen. Since bradykinin and thrombin appear to share the same signal transduction pathways, the asynchronous patterns may be determined by cell-to-cell variation in receptor number. Consistent with this notion, the latency to the onset of the responses decreases with increasing bradykinin concentration.

5 Types of Ca^{2+} Signals in Fibroblasts Stimulated with Different Classes of Mitogens

Since the introduction of methods for studying intracellular Ca^{2+} in fibroblasts, there have been numerous studies reporting changes in $[Ca^{2+}]_i$ in response to various growth factors. The mitogen-induced Ca^{2+} responses generally fall into three categories: (1) mitogens which act alone to give a strong Ca^{2+} response which is biphasic in nature; (2) mitogens which act alone to give a strong response which has only one component of the response; (3) mitogens which do not give a Ca^{2+} response when added alone but can synergize with other mitogens to give a stronger response than that resulting from the addition of either mitogen alone.

A number of mitogens fall into the first class, the members of which stimulate a biphasic response. For example, the stimulation of cultured human fibroblasts with bradykinin (Byron and Villereal 1989) produces a rapid transient followed by a rapid decline to a sustained plateau region. The rapid transient appears to result from mobilization of intracellular Ca^{2+}, since bradykinin addition in the absence of extracellular Ca^{2+} will give a transient peak which is about 85% of the size seen in the presence of external Ca^{2+}. Thus, the majority of the initial response results from mobilization of internal Ca^{2+}, with only a slight contribution from Ca^{2+} influx. The plateau region of the response is clearly due to Ca^{2+} influx since it is lost in the absence of external Ca^{2+} or can be blocked by the addition of agents such as nickel or cobalt which are known to block Ca^{2+} channels.

In general, growth factors which are known to be coupled to phospholipase C via G proteins are found to give this biphasic type of Ca^{2+} response. For example, stimulation of Swiss 3T3 cells with vasopressin (Morris et al. 1984), cultured vascular smooth muscle with angiotensin II or vasopressin (Nabika et al. 1985), cultured endothelial cells with bradykinin or angiotensin II (Morgan-Boyd et al. 1987), CHO cells transfected with platelet-derived growth factor (PDGF) receptors by PDGF (Escobedo et al. 1988) all give a transient peak of Ca^{2+} followed by a stable plateau which lasts for at least 10–15 min.

Other mitogens give only a monophasic change in Ca^{2+}. This can occur either by stimulating a Ca^{2+} influx alone or by stimulating a Ca^{2+} mobilization alone. For example, in most studies with epidermal growth factor (EGF) an increase in Ca^{2+} is observed which depends on the presence of external Ca^{2+}. Although the Ca^{2+} response to EGF resembles the transient peak to bradykinin seen in the absence of external Ca^{2+}, the EGF signal is not due to mobilizing internal Ca^{2+} pools. For example, in A431 cells (Moolenaar et al. 1986), in Swiss 3T3 cells (Hesketh et al. 1985)

and in cultured human fibroblasts (Palfrey et al. 1987) the removal of external Ca^{2+} eliminates the Ca^{2+} response. This suggests that EGF increases $[Ca^{2+}]_i$ by regulating a Ca^{2+} influx pathway which allows for Ca^{2+} entry. Another mitogen which has been suggested to act by modifying the level of Ca^{2+} influx is prostaglandin E_1 (PGE_1). In Swiss 3T3 cells, the addition of PGE_1 causes a substantial increase in $[Ca^{2+}]_i$ which is virtually eliminated by the removal of external Ca^{2+} (Yamashita and Takai 1987).

There are also reports of growth factors which cause the initial mobilization of internal Ca^{2+} pools without affecting the influx of external Ca^{2+}. For example, the addition of thrombin to cultured human fibroblasts causes a rapid transient Ca^{2+} response with no plateau region (Hendey et al. 1989). Our laboratory has studied thrombin and bradykinin responses on the same field of cells and find that cells which do not give a plateau for thrombin can subsequently respond to bradykinin stimulation with a plateau region. The sequence of addition of thrombin and bradykinin is not important (Byron and Villereal, unpublished observations).

In addition to differences in response with different growth factors, differences in the type of response to the same growth factor have been reported in different cell types. For example, it was reported that the addition of bradykinin to NRK-49F cells causes a transient Ca^{2+} response with no plateau. This response was not very different in the presence or absence of external Ca^{2+} (Marks et al. 1988). Also, it was reported that the addition of EGF alone to NRK-49F cells has no measurable effect on the intracellular Ca^{2+} concentration (Marks et al. 1988). Furthermore, in Swiss 3T3 cells the addition of vasopressin gave a transient peak but no plateau for the Ca^{2+} response (Hesketh et al. 1985), which is in contrast to the biphasic responses to vasopressin discussed above.

In contrast to the above reports, where the simple addition of a mitogen to cells causes a Ca^{2+} response which is either monophasic or biphasic, there are descriptions of Ca^{2+} responses to mitogens which are fairly complicated in nature. For example, in the NRK-49F cells, there is a complex interaction between EGF and bradykinin which has not been reported in other cells. While addition of EGF alone gives no Ca^{2+} response and the addition of bradykinin alone gives a transient peak but no plateau, the addition of EGF and bradykinin together give a Ca^{2+} response which has both a transient peak and a sustained plateau (Marks et al. 1988). The mechanism for the synergism between these two agents is not presently known.

Another example of a Ca^{2+} response which occurs only under a complex set of circumstances is the insulin-like growth factor type 2 (IGF-II) receptor activation in Balb/c 3T3 cells. IGF-II is known to be a mitogen

of Balb/c 3T3 cells but only for cells that have been rendered competent with PDGF (Stiles et al. 1979; Scher et al. 1979) and have been treated with the progression factor EGF (Leof et al. 1982). Nishimoto et al. (1987) have studied the effect of IGF-II on Balb/c 3T3 cells that are either quiescent, made competent with PDGF, or made competent with PDGF followed by treatment with the progression factor EGF. They observed that addition of IGF-II gave no Ca^{2+} response in quiescent cells or in cells made competent with PDGF. However, if PDGF-treated cells are subsequently treated with EGF for 10 min and then treated with IGF-II, a large increase in $[Ca^{2+}]_i$ is observed. The Ca^{2+} rise is transient in nature and depends totally on the presence of external Ca^{2+}.

6 Mechanisms for Agonist-Induced Elevation of $[Ca^{2+}]_i$

We have discussed the various types of Ca^{2+} responses which can be observed in mitogen-treated fibroblasts. However, the case of growth factors activating proliferating cells is really only a subset of a larger area of investigation, that is, the agonist stimulation of a Ca^{2+} signal in any cell type capable of generating this type of response. Therefore, we will spend a considerable amount of time discussing the mechanisms by which agonists, interacting with cellular receptors, can lead to either a mobilization of intracellular Ca^{2+} or an influx of Ca^{2+} through channels present in the plasma membrane.

6.1 Mobilization of Ca^{2+} from Intracellular Stores

One pathway for release of intracellular Ca^{2+} that has been established in many cell types involves the metabolism of the membrane phospholipid, phosphatidyl inositol 4,5-bisphosphate (PIP_2), by a specific phosphodiesterase, phospholipase C (PLC; reviewed by Berridge and Irvine 1989). This enzyme can be activated by receptor-mitogen interactions and catalyzes the hydrolysis of PIP_2 into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) (this process will hereafter be referred to as receptor-mediated PI turnover). $Ins(1,4,5)P_3$ binds to a receptor associated with an intracellular Ca^{2+} store and releases the stored Ca^{2+} by opening a channel in the membrane of that organelle. DAG is an activator of protein kinase C (PKC) and may be involved in modulation of Ca^{2+} signals.

6.1.1 PI Turnover in Ca^{2+} Regulation

Our understanding of the role of inositol phosphates in the regulation of Ca^{2+} signals in the growth factor signal transduction process is based largely on techniques developed in nonproliferating cells which were then adapted to study this question in proliferating cells. For years, investigators had interpreted the hormonal stimulation of $^{45}Ca^{2+}$ efflux from kidney cell, liver cells, and fibroblasts to mean that an important component of receptor stimulation was the release of Ca^{2+} from internal stores. Although Ca^{2+} storage in specialized organelles in muscle and the mechanisms for release of this Ca^{2+} have been recognized for many years, a comparable understanding of Ca^{2+} storage in nonmuscle cells has only recently been obtained. This understanding arose principally from the work of Michell (see Michell 1975) and Berridge (see Berridge 1987) who investigated the physiological importance of the PI turnover pathway. With the demonstration that PLC cleaves PIP_2 to produce Ins(1,4,5)P_3 and DAG, a search began for the physiological significance of the released Ins(1,4,5)P_3. This was soon discovered and reported in a study by Streb et al. (1983). In this study they permeabilized pancreatic acinar cells and studied the uptake of Ca^{2+} into internal pools and investigated the effect of Ins(1,4,5)P_3 on the release of Ca^{2+} from these pools. They demonstrated that Ca^{2+} can be taken up into a nonmitochondrial pool in the presence of ATP and that the addition of Ins(1,4,5)P_3 would release the Ca^{2+} from this pool. Studies in other cell types soon supported this mechanism of action of Ins(1,4,5)P_3. There is now considerable evidence supporting the fact that mitogens such as bombesin (Heslop et al. 1986; Wakelam et al. 1986), bradykinin (Jamieson and Villereal 1987), PDGF (Nanberg and Rozengurt 1988), and vasopressin (Jamieson and Villereal 1987) can stimulate PLC in fibroblasts with the subsequent release of Ins(1,4,5)P_3 and mobilization of intracellular Ca^{2+} stores (Muldoon et al. 1987; Etscheid and Villereal 1989).

6.1.2 Metabolism and Function of Inositol Phosphates

Since there have been several recent reviews (Berridge and Irvine 1989; Nishizuka 1988; Fain 1990) on PLC/PIP_2 we will not go into any detail concerning the mechanism for the release of inositol phosphates. However, because of their potential importance in the regulation of $[Ca^{2+}]_i$, we will briefly discuss the various forms of the water-soluble inositol phosphates which are released upon breakdown of PIP_2 or are produced upon metabolism of Ins(1,4,5)P_3. The initial ion exchange methods for identifying the inositol phosphate products released followed hormonal stimulation of

cells were able to resolve three major products, IP, IP_2, and IP_3. The pattern of release of these three components and the changes in the PI pools themselves argued that hormonal stimulation produced IP_3 which was soon broken down to IP_2 and IP. However, with the introduction of HPLC methods to the identification of the inositol phosphates, it became clear that matters were much more complicated than was initially thought. It was demonstrated that there existed two species of inositol phosphates, Ins(1,4)P_2 and Ins(3,4)P_2 under the IP_2 peak identified on ion exchange columns. In addition, there were several species in the ion exchange elution peak previously identified as IP_3, namely Ins(1,4,5)P_3, Ins(1,3,4)P_3, and Ins(1,3,4,5)P_4. With the identification of these additional forms of inositol phosphates, a vigorous investigation was undertaken to determine the physiological significance of these compounds. Of particular interest was the question of whether any of these compounds besides Ins(1,4,5)P_3 would be able to mobilize intracellular Ca^{2+}.

Recently, more highly phosphorylated forms of inositol have been detected. Of these, only Ins(1,3,4,5)P_4 is currently postulated to have some physiological significance. We now know that Ins(1,3,4,5)P_4 is produced via the action of a Ins(1,4,5)P_3 kinase. This kinase has been discovered as a soluble enzyme present in brain (Irvine et al. 1986), but has since been reported in liver (Hansen et al. 1986), parotid (Hawkins et al. 1986), lymphocytes (Steward et al. 1986), and insulinoma cells (Biden and Wollheim 1986). This kinase is specific for the 3-position of Ins(1,4,5)P_3 and produces Ins(1,3,4,5)P_4 from only this isomer of InsP_3.

The description of a kinase which phosphorylated Ins(1,4,5)P_3 led to rampant speculation concerning the role of this phosphorylation pathway. Did the phosphorylation of Ins(1,4,5)P_3 serve as an inactivation pathway whereby Ins(1,4,5)P_3 was inactivated by phosphorylation, or was the Ins(1,3,4,5)P_4 produced an important physiological messenger. One inactivation pathway for Ins(1,4,5)P_3 had already been described whereby a phosphatase specific for Ins(1,4,5)P_3 would remove the phosphate from the 5 position, resulting in the production of Ins(1,4)P_2. The phosphorylation of Ins(1,4,5)P_3 by Ins(1,4,5)P_3 kinase could be a parallel pathway for the removal of the active form of Ins(1,4,5)P_3. This seems like a valid possibility since Ins(1,3,4,5)P_4 is metabolized to the apparently inactive isomer Ins(1,3,4)P_3. While the early studies did not decide the issue of whether Ins(1,3,4,5)P_4 was an important physiological mediator, they did resolve the issue of the source of the Ins(1,3,4)P_3, which is now known to come from the action of a 5-phosphatase acting on Ins(1,3,4,5)P_4.

At present, it is thought that Ins(1,3,4,5)P_4 might be involved in the regulation of membrane Ca^{2+} channels in response to hormonal stimulation of cells. This belief comes from a study in sea urchin eggs in which

$Ins(1,3,4,5)P_4$ was microinjected and was observed to cause an immediate elevation of the fertilization envelope for eggs in the presence, but not in the absence, of external Ca^{2+} (Irvine and Moore 1986). This process was somewhat complicated since in order to see the effect of $Ins(1,3,4,5)P_4$, the investigators had to coinject $Ins(2,4,5)P_3$, an isomer that cannot be phosphorylated to produce IP_4, but is known to mobilize Ca^{2+} from internal stores. Since $Ins(2,4,5)P_3$ does not cause elevation of the fertilization envelope on its own, it is clear that the $Ins(1,3,4,5)P_4$ must be playing some physiological role.

It has been speculated that the synergism between the Ca^{2+}-mobilizing agent $Ins(2,4,5)P_3$ and $Ins(1,3,4,5)P_4$ may be related to an effect of $Ins(1,3,4,5)P_4$ on the reloading of the $Ins(1,4,5)P_3$-sensitive Ca^{2+} store via an entry of Ca^{2+} across the plasma membrane. This speculation comes from the theory that vesicles reload with Ca^{2+} via a specialized network by which Ca^{2+} enters the storage compartments without coming directly through the cytoplasm (Putney 1986). The proposed synergism between $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ is supported by patch clamp studies in mouse lacrimal acinar cell. Whole cell perfusion studies indicate that both $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ are necessary to see Ca^{2+}-activated K^+ currents (Morris et al. 1987). A role for $Ins(1,3,4,5)P_4$ in the control of Ca^{2+} fluxes is also suggested by Irvine and his colleagues (Irvine et al. 1988). This is supported by the observation that the presence of $Ins(1,3,4,5)P_4$ will enhance the $Ins(1,4,5)P_3$-induced release of Ca^{2+} from internal stores in microsomal fractions from pituitary cells (Spat et al. 1987). The hypothesis that the generation of $Ins(1,3,4,5)P_4$ is important in changing the Ca^{2+} response from a transient one to a more sustained one is consistent with the observation that the activity of $Ins(1,4,5)P_3$ kinase is stimulated by Ca^{2+} via a calmodulin-dependent mechanism (Ryu et al. 1987; Morris et al. 1988). Therefore, the short burst of Ca^{2+} in response to release of $Ins(1,4,5)P_3$ can lead to an increased production of $Ins(1,3,4,5)P_4$ which can in turn lead to a sustained influx of Ca^{2+}.

6.1.3 $Ins(1,4,5)P_3$-Independent Mechanisms for Mobilizing Intracellular Ca^{2+}

Our understanding of the mechanisms of agonist-stimulated release of intracellular Ca^{2+} stores has been complicated by the recent discovery of agents which are capable of releasing Ca^{2+}, apparently by interacting at some intracellular site(s) other than the $Ins(1,4,5)P_3$ receptor. These agents, arachidonic acid (Wolf et al. 1986) and sphingosine (Ghosh et al. 1990), may be generated and released into the cytosol by agonist-receptor interactions and may therefore participate in agonist-induced changes in

$[Ca^{2+}]_i$. In permeabilized cells and isolated microsomal vesicles, Gill et al. (1988) have found that guanine nucleotides can release sequestered Ca^{2+} from $Ins(1,4,5)P_3$-sensitive and $Ins(1,4,5)P_3$-insensitive stores, although the mechanisms underlying these effects remain to be elucidated. Recent reports have also revealed that thapsigargin, a nonphorbol ester type tumor promoter, when applied extracellularly, can release intracellular Ca^{2+} stores without elevating PI turnover (Jackson et al. 1988; Takemura et al. 1989).

Another new mechanism for release of intracellular Ca^{2+} from internal stores has recently been described by Lee's laboratory (Clapper and Lee 1985; Clapper et al. 1987; Dargie et al. 1990). They have identified a metabolic product of NAD that has potent Ca^{2+} releasing activity when measured in homogenates of sea urchin eggs. The metabolite was initially referred to as E-NAD (for enzyme-activated NAD). They found that incubation of NAD with high-speed supernatants from sea urchin eggs would lead to the production of E-NAD. Intracellular Ca^{2+} was found to be mobilized by microinjection of high performance liquid chromatography (HPLC) purified E-NAD into sea urchin eggs. Lee et al. (1988) recently identified the structure of E-NAD to be that of cyclic ADP-ribose. Much work remains before the mechanism for the control of the synthesis of this compound in sea urchin eggs is understood and to determine whether it is a physiologically important regulator. The enzyme which catalyzes the production of cyclic ADP-ribose has been discovered in heart and liver which suggests that this also may be an important pathway for mobilizing intracellular Ca^{2+} in mammalian tissues.

Another postulated mechanism for agonist-stimulated Ca^{2+} release may be analogous to the excitation-contraction coupling mechanism of Ca^{2+} release from sarcoplasmic reticulum in muscle. In this case, an $Ins(1,4,5)P_3$-insensitive Ca^{2+} store is filled with Ca^{2+} until some threshold is reached which causes the release of the stored Ca^{2+} into the cytosol. Involvement of this type of Ca^{2+} pool has been invoked by Berridge et al. (1988) to explain oscillations of $[Ca^{2+}]_i$ which have been observed in many cell types following physiological stimulation. This Ca^{2+}-sensitive Ca^{2+} release pathway may be activated by caffeine, in analogy to the muscle Ca^{2+} store, but not by $Ins(1,4,5)P_3$. Caffeine-sensitive Ca^{2+} stores have been observed also in nonmuscle cells (Cheek et al. 1990; Kuba 1980).

6.2 Mitogen-Induced Ca^{2+} Entry

Agonist-stimulated Ca^{2+} entry across the plasma membrane is less well characterized than release of intracellular Ca^{2+} stores. Ca^{2+} entry mecha-

nisms vary among different cell types and depend on the agonist used to stimulate the cells. Voltage-sensitive Ca^{2+} channels have been found in nonexcitable cells (Chen et al. 1988), but the contribution of this influx pathway to mitogen-stimulated $[Ca^{2+}]_i$ elevation is uncertain. In most cases Ca^{2+} influx appears to be dependent on continued receptor occupation by its specific ligand. This suggests the possibility of receptor-operated Ca^{2+} channels similar to the nicotinic acetylcholine receptor channel and the N-methyl-D-aspartate (NMDA) receptor channel, although no such mitogen receptor channel has been definitively identified. Another possibility is an indirect coupling of receptor and Ca^{2+} channel whereby opening of the Ca^{2+} channel is dependent on some diffusible signal which is generated by receptor occupation and is rapidly removed when the receptor is unoccupied. The existence of such a Ca^{2+} entry pathway is suggested by a measurable time lag prior to influx following stimulation of cells with some agonists (Sage and Rink 1987; Rink and Sage 1988). A single agonist may activate more than one Ca^{2+} entry pathway, as recently reported for bovine aortic endothelial cells (Schilling et al. 1989) and platelets (Sage et al. 1990).

Recent data from our laboratory indicates that mitogens such as EGF and bradykinin may regulate voltage-sensitive Ca^{2+} channels in cultured human fibroblasts. Based on earlier studies demonstrating L-type voltage-sensitive Ca^{2+} channels in cultured fibroblasts (Chen et al. 1988), we began to study whether this type of Ca^{2+} channel could be regulated by growth factors. We observed that utilizing $^{45}Ca^{2+}$ flux studies we could demonstrate influx via a nitrendipin sensitive channel (K_i of 1 n*M*) which could be stimulated by EGF and bradykinin (Baumgarten et al. 1991).

6.3 Link Between Ca^{2+} Mobilization and Ca^{2+} Entry

A great deal of recent research has been devoted to examining the relationship between Ca^{2+} mobilization and Ca^{2+} entry. Although much of this work was done in nonproliferating cells with agonists other than growth factors, it is important to review this work because the same issues arise when one asks how Ca^{2+} entry in growth factor-stimulated fibroblasts is controlled.

6.3.1 Capacitative Ca^{2+} Entry Model

A model developed by J.W. Putney Jr. (1986) suggests that agonist-induced Ca^{2+} entry in parotid acinar cells is regulated by the Ca^{2+} content of the intracellular Ca^{2+} stores. This model arose largely from some

earlier work from Putney's lab which examined the emptying and refilling of the intracellular Ca^{2+} stores in parotid cells. Since these cells possess Ca^{2+}-activated K^+ channels, Aub et al. (1982) used ^{86}Rb efflux as an indirect measure of cytosolic $[Ca^{2+}]$, due to the inability at that time to directly measure $[Ca^{2+}]_i$. They found that, in Ca^{2+}-free medium, carbachol elicited a rapid transient elevation of ^{86}Rb efflux which they attributed to the release of intracellular Ca^{2+} stores. In Ca^{2+}-containing medium, carbachol produced a sustained elevation of ^{86}Rb efflux which they suggested was the result of increased Ca^{2+} entry. In order to examine the emptying and refilling of the intracellular Ca^{2+} stores, they stimulated the cells with carbachol in Ca^{2+}-free medium and then terminated the carbachol action by the addition of the muscarinic receptor antagonist atropine. When these cells were maintained in Ca^{2+}-free medium and subsequently exposed to substance P, no further elevation of ^{86}Rb efflux was observed. Since substance P had been previously shown to mobilize intracellular Ca^{2+} via PI turnover in these cells, this was interpreted as an indication that the $Ins(1,4,5)P_3$-releasable intracellular Ca^{2+} stores had been depleted by the prior exposure to carbachol in Ca^{2+}-free medium. If extracellular Ca^{2+} was added for a 2-min period between carbachol and substance P treatments, the stores refilled, as evidenced by a transient elevation of ^{86}Rb efflux in response to substance P. The magnitude of this response depended on the concentration of Ca^{2+} added during the 2-min period.

Several observations were made from these experiments: (1) it was apparent that the refilling of the intracellular Ca^{2+} stores required extracellular Ca^{2+}; (2) the refilling process was rapid, occurring in less than 2 min, even though they had observed no rapid depletion of the intracellular Ca^{2+} stores when unstimulated cells were maintained in Ca^{2+}-free medium; (3) Ca^{2+} was apparently entering to refill the intracellular stores at a time when the agonist action had been terminated by addition of antagonist and, therefore, in the absence of receptor-generated messengers; (4) refilling of the stores occurred without a detectable elevation of $[Ca^{2+}]_i$ (as measured by ^{86}Rb efflux).

Putney's model endeavored to explain these observations. He proposed that the $Ins(1,4,5)P_3$-releasable intracellular Ca^{2+} stores are located in close apposition to the plasma membrane. In the event of agonist-mediated PI turnover, elevated $Ins(1,4,5)P_3$ levels trigger the release of Ca^{2+} from these stores into the cytosol. This lowers the Ca^{2+} content within the stores and relieves inhibition of a Ca^{2+} entry pathway in the adjacent plasma membrane. Ca^{2+} then enters through this pathway across the plasma membrane and into the intracellular stores, bypassing the bulk of the cytosol. While $Ins(1,4,5)P_3$ levels remain elevated, the Ca^{2+} which enters

the intracellular stores will leak into the cytosol, accounting for the sustained elevation of $[Ca^{2+}]_i$ observed during agonist stimulation in Ca^{2+}-containing medium. When $Ins(1,4,5)P_3$ levels fall (as on termination of agonist binding to receptor), the intracellular release channel closes, but the entry pathway across the plasma membrane remains open and Ca^{2+} can enter to refill the intracellular stores. As the Ca^{2+} content of the stores rises, the entry pathway is inhibited until, when the stores are full, no further Ca^{2+} entry occurs.

This model is appealing for a number of reasons. Since the Ca^{2+} content of the intracellular stores controls the entry of Ca^{2+} across the plasma membrane (Putney coined the phrase "capacitative Ca^{2+} entry" to describe this phenomenon), a single messenger, $Ins(1,4,5)P_3$, can regulate both the release of intracellular Ca^{2+} and the entry of Ca^{2+} from the extracellular space. Furthermore, a close physical association of the $Ins(1,4,5)P_3$-releasable intracellular Ca^{2+} stores with the plasma membrane makes intuitive sense from the point of view that receptor-mediated PI turnover occurs at the plasma membrane and, therefore, the formation of $Ins(1,4,5)P_3$ occurs close to its site of action. (Recent evidence for a close physical association of the intracellular Ca^{2+} stores with the plasma membrane is discussed below.) An earlier report by Poggioli et al. (1985) had suggested that a reduction of $[Ca^{2+}]$ on the inner surface of the plasma membrane could increase the permeability of that membrane to Ca^{2+}. Putney's model elaborated on that finding to speculate that, if the intracellular Ca^{2+} stores were located adjacent to the plasma membrane, then depleting these stores of Ca^{2+} might reduce $[Ca^{2+}]$ on the inner surface of the plasma membrane and increase the permeability of that membrane to Ca^{2+} as described by Poggioli et al. (1985). Thus, as Putney noted, the model was constructed without making any particularly unreasonable assumptions.

6.3.2 Testing the Capacitative Ca^{2+} Entry Model

Since Putney's model was first published, many reports relating to Ca^{2+} entry and refilling of the intracellular Ca^{2+} stores have appeared in the literature (reviewed by Taylor 1990). Most recent work has utilized fura2 as a more sensitive indicator of $[Ca^{2+}]_i$. An early report by Merritt and Rink (1987) used fura2-loaded parotid acinar cells to arrive at very similar conclusions to Putney. These authors suggested that a gap junction-like channel might exist between the plasma membrane and the intracellular Ca^{2+} stores and that Ca^{2+} entry through this channel would be regulated by the Ca^{2+} content of the stores. More recent studies using fura2 have continued to support the capacitative Ca^{2+} entry model. Takemura and

Putney (1989) found that when fura2-loaded parotid cells were stimulated with methacholine in Ca^{2+}-free medium followed by atropine treatment, the addition of extracellular Ca^{2+} produced an elevation of $[Ca^{2+}]_i$. They noted that this elevation was larger than that produced by Ca^{2+} addition to unstimulated cells which had been incubated in Ca^{2+}-free medium. They also showed that this increased elevation of $[Ca^{2+}]_i$ on Ca^{2+} addition was observed as late as 20 min after atropine addition, but it was not observed if the intracellular stores were first allowed to refill. Putney et al. (1989) and Takemura et al. (1989) found that thapsigargin releases the intracellular Ca^{2+} stores of parotid acinar cells without elevating Ins(1,4,5)P_3 levels, and stimulates Ca^{2+} entry, apparently by the same pathway as is stimulated by methacholine in these cells. This was interpreted as evidence that it was the depletion of the intracellular Ca^{2+} stores rather than some other signal (i.e., inositol phosphates) that controls the entry of Ca^{2+}.

Although very few studies have appeared which have examined this phenomenon in individual cells, there are some data from individual cells which support the capacitative model. Refilling of intracellular Ca^{2+} stores in parietal cells (Negulescu and Machen 1988) and human endothelial cells (Jacob et al. 1988; Rink and Hallam 1989; Jacob 1990) can occur without a measurable elevation of $[Ca^{2+}]_i$, a finding consistent with a pathway for Ca^{2+} entry which does not traverse the cytosol. A recent study by Jacob (1990) has examined divalent cation entry into single human umbilical vein endothelial cells. Earlier work had shown that both histamine and thrombin stimulate Ca^{2+} entry into these cells (Hallam et al. 1988, 1989). Those studies had utilized Mn^{2+}-induced quench of fura2 fluorescence as an indicator of Ca^{2+} entry, assuming that Mn^{2+} enters the cells through the same pathway as Ca^{2+}. Jacob expanded on that earlier work by examining the effects of histamine-induced release of intracellular Ca^{2+} stores and the refilling of these stores on Mn^{2+} entry. He found that when Mn^{2+} is added during histamine stimulation, Mn^{2+} entry coincides with the release of intracellular Ca^{2+}. Mn^{2+} entry also occurs when Mn^{2+} is added after a brief exposure of the cells to histamine to deplete the intracellular Ca^{2+} stores. However, if the stores are allowed to refill by a transient exposure to extracellular Ca^{2+} before Mn^{2+} addition, no Mn^{2+} entry is observed, suggesting that the pathway for Mn^{2+} entry is only open when the intracellular stores are depleted of Ca^{2+}. He expanded on this observation by examining the rate of Mn^{2+} entry with varying fullness of the stores. The fullness of the stores was varied both by adding varying amounts of Ca^{2+} back to depleted stores and by partially depleting the stores with varying exposures to histamine. With either of these procedures, he found that the rate of Mn^{2+} entry varies inversely with the

Ca^{2+} content of the intracellular stores. As noted by Jacob, these findings are clearly consistent with a model of Ca^{2+} entry regulated by the Ca^{2+} content of the intracellular stores.

Further support for Putney's model has come from studies which have examined the localization of intracellular Ca^{2+} stores in acinar cells. Haase et al. (1984) used electron microscopy techniques to examine pancreatic acinar cells and found that cholecystokinin and carbachol stimulation reduced by 80% the Ca^{2+} content of stores located along the plasma membrane. Foskett et al. (1989) combined electrophysiological measurements with fura2 fluorescence measurements of $[Ca^{2+}]_i$ to compare the kinetics of agonist-induced membrane hyperpolarization with the elevation of $[Ca^{2+}]_i$ in parotid acinar cells. These cells have Ca^{2+}-sensitive K^+ channels in their basolateral membranes. A rapid agonist-stimulated membrane hyperpolarization is believed to result from an elevation of $[Ca^{2+}]_i$, which subsequently activates these channels. Foskett et al. found that simultaneous measurements of $[Ca^{2+}]_i$ and membrane potential revealed that membrane hyperpolarization preceded the fura2-detectable elevation of $[Ca^{2+}]_i$. In order to determine if the hyperpolarization might be due to localized changes in $[Ca^{2+}]_i$ which are not detected by fura2, they loaded the cells with the Ca^{2+} chelator, dimethyl-BAPTA, and again simultaneously measured $[Ca^{2+}]_i$ and membrane potential following carbachol stimulation. In Ca^{2+}-free medium, the dimethyl 1,2-bis(2-aminophenoxyethane)-N-N-N′-N′-tetraacetic acid (BAPTA) loaded cells exhibited no carbachol-stimulated hyperpolarization or $[Ca^{2+}]_i$ elevation, unless the internal stores were allowed to refill by a brief exposure to extracellular Ca^{2+}. In this case, carbachol elicited a rapid hyperpolarization even in the absence of a detectable elevation of $[Ca^{2+}]_i$, suggesting that a localized release of intracellular Ca^{2+} stores near the basolateral membrane could activate K^+ channels without elevating overall cytosolic $[Ca^{2+}]$.

Despite the general agreement that the Ca^{2+} content of the intracellular stores might be regulating Ca^{2+} entry, a substantial body of evidence was amassed which indicated that Ca^{2+} entry was occurring via a cytosolic route rather than through the restricted pathway originally envisaged by Putney or the gap junction-like channel of Merritt and Rink. With the use of fura2, it was apparent that, in many different cell types, addition of extracellular Ca^{2+} to cells which had been depleted of Ca^{2+} by transient exposure to agonist in Ca^{2+}-free medium resulted in an elevation of $[Ca^{2+}]_i$ (Pandol et al. 1987; Takemura and Putney 1989; Hallam et al. 1989; Kwan and Putney 1990; Shuttleworth 1990). This suggested that some Ca^{2+} was entering the cytosol before it was taken up by the intracellular stores. Furthermore, the rate of Ca^{2+} entry was found to be greater than the rate of release of intracellular Ca^{2+} stores in pancreatic

cells (Muallem et al. 1988b) and avian nasal gland cells (Shuttleworth 1990), an observation inconsistent with the notion that entering Ca^{2+} must pass through the intracellular stores before it reaches the cytosol. Perhaps the most convincing evidence arguing against a direct pathway for Ca^{2+} entry into the intracellular stores comes from a study by Kwan and Putney (1990) which examined the entry of Ba^{2+} and Sr^{2+} and refilling of intracellular Ca^{2+} stores in lacrimal acinar cells. These authors found that a Ca^{2+} entry pathway permeable to both Ba^{2+} and Sr^{2+} was activated when the intracellular Ca^{2+} stores were released. They further noted that Sr^{2+} which enters by this route is taken up by the intracellular stores and can be released by subsequent exposure to agonist, but Ba^{2+}, which is a poor substrate for the store's Ca^{2+} pump, does not enter the stores. Thus, it would appear that these divalents enter the cytosol directly and that Ca^{2+} and Sr^{2+} can then be pumped into the stores where they are then available for release by agonist. Ba^{2+} remains in the cytosol, incapable of entering the stores via the pump.

The current state of the capacitative Ca^{2+} entry model is that the Ca^{2+} content of the intracellular stores controls the entry of Ca^{2+} across the plasma membrane into the cytosol, perhaps acting at some distance from the entry site by releasing some diffusible messenger (Putney et al. 1989; Takemura et al. 1989; Taylor 1990). Variations of this model proposed by Irvine (1989, 990) have attempted to incorporate a role for inositol 1,3,4,5-tetrakisphosphate ($Ins(1,3,4,5)P_4$). This metabolite of $Ins(1,4,5)P_3$ formed by the action of a Ca^{2+}/calmodulin-sensitive $Ins(1,4,5)P_3$-3-kinase, has been implicated in agonist-mediated Ca^{2+} mobilization (Irvine et al. 1988). One model proposed by Irvine (1989) suggests that $Ins(1,3,4,5)P)_4$ may be acting to regulate communication between multiple intracellular Ca^{2+} stores which in turn are connected to the plasma membrane by a gap junction-like channel. This model seeks to combine a number of disparate pieces of evidence by including calciosomes (Volpe et al. 1988), a possible role for GTP in regulating the size of releasable intracellular Ca^{2+} stores (Mullaney et al. 1987), a role for $Ins(1,3,4,5)P_4$, and a capacitative Ca^{2+} entry mechanism. This complicated and highly speculative model has recently been followed by another equally speculative model (Irvine 1990) which suggests that an $Ins(1,3,4,5)P_4$ receptor on the plasma membrane is physically associated with the $Ins(1,4,5)P_3$ receptor on the endoplasmic reticulum and that $Ins(1,3,4,5)P_4$, combined with the Ca^{2+} content of the endoplasmic reticulum, can alter the conformation of this complex to activate a Ca^{2+} channel in the plasma membrane.

Depending on the cell type, depletion of the intracellular Ca^{2+} stores may not be sufficient to stimulate Ca^{2+} entry. For example, Kass et al. (1989) used 2,5-di-(tert-butyl)-1,4-benzohydroquinone (tBuBHQ), a specif-

ic inhibitor of liver microsomal ATP-dependent Ca^{2+} sequestration, to investigate intracellular Ca^{2+} homeostasis in rat hepatocytes. This compound was shown to deplete the Ins(1,4,5)P_3-sensitive intracellular Ca^{2+} stores without elevating Ins(1,4,5)P_3 levels. Depletion of these stores with tBuBHQ blocked further release of intracellular Ca^{2+} by vasopressin, but did not stimulate Ca^{2+} entry or inhibit vasopressin-stimulated Ca^{2+} entry. These results suggest that depleting the intracellular Ca^{2+} stores is not sufficient to stimulate Ca^{2+} entry in hepatocytes, although the authors concede that they cannot rule out inhibition by tBuBHQ of Ca^{2+} entry by a mechanism such as that described by Poggioli et al. (1985).

Another recent report (Stauderman and Pruss 1989) has concluded that angiotensin II (A_{II}) stimulates Ca^{2+} entry into bovine adrenal chromaffin cells by a receptor-linked mechanism other than the release of the intracellular Ca^{2+} stores, i.e., by a noncapacitative route. Stauderman and Pruss, using fura2-loaded chromaffin cells, showed that A_{II} produces a biphasic elevation of $[Ca^{2+}]_i$ in these cells which can be attributed to a combination of Ca^{2+} entry and release of intracellular Ca^{2+} stores. They utilized the Ca^{2+} ionophore ionomycin to deplete the intracellular Ca^{2+} stores and found that subsequent addition of A_{II} produced an elevation of $[Ca^{2+}]_i$ which was dependent on extracellular Ca^{2+} and was inhibitable by the nonspecific Ca^{2+} channel blocker La^{3+}. They concluded that A_{II} was stimulating Ca^{2+} entry at a time when the intracellular Ca^{2+} stores had already been depleted by ionomycin and that, therefore, the stimulus for activating this entry pathway was not the emptying of the stores.

While these findings might imply that capacitative Ca^{2+} entry is not a universal phenomenon, we might reconcile the results of Kass et al. and Stauderman and Pruss with the capacitative Ca^{2+} entry data by speculating that agonists activate more than one Ca^{2+} entry pathway – one into the cytosol which requires receptor occupation for continued activity, and another which is capacitative, allowing Ca^{2+} to enter into a restricted space where it is taken up into the intracellular stores without traversing the bulk of the cytosol. This possibility has recently received strong support by the work of Sage et al. (1990) who examined ADP-stimulated Ca^{2+} entry in human platelets. They had previously shown that, at 37 °C, ADP stimulates Ca^{2+} entry into platelets with a delay of less than 20 ms, while the release of intracellular Ca^{2+} has a slower onset (approx. 200 ms; Sage and Rink 1987). The more recent study utilized Mn^{2+} quench of fura2 fluorescence as an indicator of Ca^{2+} entry into platelets at 17 °C. At this lower temperature, they resolved a biphasic elevation of $[Ca^{2+}]_i$ evoked by ADP. With Mn^{2+}, they determined that there was a rapid phase of entry which was unaffected by the temperature change and a delayed phase of entry which coincided with the release of intracellular

Ca^{2+}. Forskolin blocked the release of intracellular Ca^{2+} and the delayed phase of Ca^{2+} entry, but not the rapid phase of entry. Sage et al. concluded that the delayed phase of Ca^{2+} entry is regulated by the release of the intracellular Ca^{2+} stores. The more rapid phase of Ca^{2+} entry they attributed to a receptor-operated channel, a finding recently confirmed by single channel patch clamp recording (Mahaut-Smith et al. 1990).

Recently, our laboratory has completed a study of the link between Ca^{2+} mobilization from internal pools and Ca^{2+} entry pathways in cultured human fibroblasts. In particular we investigated the relationship between Ca^{2+} influx pathways activated by bradykinin and the state (filled or empty) of the intracellular Ca^{2+} pools. We observed that when one empties the Ins(1,4,5)P_3-sensitive Ca^{2+} pool by stimulating cells with bradykinin in Ca^{2+}-free medium, the cells must see Ca^{2+} in the absence of bradykinin for the pools to refill (Byron et al. 1992). When Ca^{2+} is added back to cells 10 min after the removal of bradykinin, there is still a stimulated Ca^{2+} influx present. This is consistent with the idea that emptying of the pools has activated a Ca^{2+} influx pathway. However, we have been able to dissociate the two phenomena and have shown that the sustained Ca^{2+} influx is due to the long-lived activation of a Ca^{2+} influx pathway which can be turned off without affecting the pathway through which the internal Ca^{2+} pools refill. For example, we have demonstrated that the divalent cation Ni^{2+} can block the Ca^{2+} influx pathway responsible for the increase in cytosolic Ca^{2+} without blocking the refilling of the internal Ca^{2+} pools (Byron et al. 1992). In addition, one can temporally separate the influx pathway from the refilling pathway.

These temporal studies emphasized the need for one to analyze the cells on an individual basis to answer this particular question of Ca^{2+} regulation. If we unload cell Ca^{2+} by treating cells with bradykinin in a Ca^{2+}-free solution and then wait for 30 min before trying to refill the internal pools, we get two different answers, depending on whether we look at the population as a whole or whether we look at individual cell responses. The data from the whole cell population indicates that there is a substantial rise in Ca^{2+} when the Ca^{2+} is added back to the cells, implying that the empty state of the pools has trapped the Ca^{2+} influx pathways in the open state. However, when we analyze individual cells we see that the Ca^{2+} rise of the population was due to Ca^{2+} influx in only about 20% of the cells. In the majority of the cells there is no rise in cytosolic Ca^{2+} and yet the internal pools do refill during this time. The longer the wait after bradykinin removal, the fewer cells show a rise in cytosolic Ca^{2+}, yet all of the cells refill their internal Ca^{2+} pools during this reloading time. Thus, the individual cell data suggest two things that are not shown in the population studies: (1) the Ca^{2+} influx pathways responsible for the large rise in cyto-

solic Ca^{2+} do not appear to remain open for as long as the internal pools are empty, and (2) there appears to be a mechanism for refilling the internal pools without an elevation of cytosolic Ca^{2+}. This latter observation lends support for the model proposed by Putney that there is a pathway for filling the internal pools which does not access the bulk cytosol. We would merely modify the model by adding a second Ca^{2+} influx pathway which is controlled perhaps by second messenger activity.

7 Oscillations of $[Ca^{2+}]_i$ in Agonist-Stimulated Cells

Oscillations of $[Ca^{2+}]_i$ have been observed in many cell types in response to mitogens and other physiological stimuli (reviewed by Berridge and Gallione 1988; Berridge 1990). The nature of the oscillations varies among different cell types and for a given cell type the shapes of Ca^{2+} transients may depend on which agonist induces the oscillations (Berridge et al. 1988; Woods et al. 1987). In several cases the frequency of $[Ca^{2+}]_i$ oscillations varies in proportion to the dose of agonist presented to the cells (Woods et al. 1987), suggesting the possibility of frequency-modulated cell responses to physiological stimuli.

The mechanics of $[Ca^{2+}]_i$ oscillations in cells are still open to speculation. Oscillations in the production of Ins(1,4,5)P_3 could produce periodic release of intracellular Ca^{2+}. Periodic formation of Ins(1,4,5)P_3 might occur based on a negative feedback loop operating through PKC, as suggested by Cobbold et al. (1988), whereby PKC reversibly phosphorylates a G-protein and inhibits the transduction of receptor binding to PLC activation. Alternatively, oscillations in Ins(1,4,5)P_3 production could result from a reciprocal coupling between Ca^{2+} and Ins(1,4,5)P_3 as described by Meyer and Stryer (1988). In this model, agonist-induced Ins(1,4,5)P_3 release leads to rapid release of Ca^{2+} that feeds back positively to increase PLC activity and produce a burst of Ins(1,4,5)P_3. Lowering of $[Ca^{2+}]_i$ by reuptake of released Ca^{2+} into the Ins(1,4,5)P_3-sensitive store and into mitochondria removes the positive feedback on PLC and Ins(1,4,5)P_3 levels fall.

Other postulated mechanisms attempt to explain how Ca^{2+} oscillations might occur in the continued presence of Ins(1,4,5)P_3. These models were developed, in part, to explain studies that have shown Ca^{2+} oscillations in photoreceptors (Payne et al. 1988) and *Xenopus* oocytes (Berridge 1988) following a single injection of Ins(1,4,5)P_3. One model would suggest that cytosolic Ca^{2+} would feedback negatively on the Ca^{2+} release channel to inhibit further release until $[Ca^{2+}]_i$ has been lowered by reuptake of Ca^{2+}

into the pool and inhibition is removed (Payne et al. 1988; Prentki et al. 1988). Another model (Berridge et al. 1988) requires the existence of a Ca^{2+}-releasable Ca^{2+} pool. According to this model, Ca^{2+} released from an Ins(1,4,5)P_3-sensitive pool would be taken up into a second Ca^{2+} pool which is Ins(1,4,5)P_3 insensitive. When the Ca^{2+} concentration in this pool reaches some threshold level, a release channel opens and Ca^{2+} is released back into the cytosol. In the continued presence of Ins(1,4,5)P_3, this process could continue cyclically as long as the Ca^{2+} concentration in the second pool continues to reach the threshold necessary for release. Clearly, in order to further discuss the basis of Ca^{2+} oscillations, we need to consider in more depth the mechanisms available for dealing with free Ca^{2+} once it enters the cell or has been released from internal pools. In the next section we will discuss the mechanisms for returning Ca^{2+} to its basal level and then return to a more detailed discussion of Ca^{2+} oscillations.

8 Mechanisms for Restoring Ca^{2+} to Basal Levels After Stimulation and Their Role in Ca^{2+} Oscillations

An examination of the regulation of cytosolic [Ca^{2+}] with regard to agonist-induced elevation of $[Ca^{2+}]_i$ must consider the homeostatic mechanisms present in the cell which tend to resist changes in $[Ca^{2+}]_i$ from resting levels. As demonstrated in the previous chapters, agonists stimulate the release of intracellular Ca^{2+} and the entry of Ca^{2+} into the cytosol from the extracellular space, producing an elevation of $[Ca^{2+}]_i$ which may be sustained during many minutes of exposure to agonist and even for some time after removal of agonist from the medium bathing the cells. However, a number of cell processes which are active in both resting and stimulated cells eventually prevail to return $[Ca^{2+}]_i$ to resting levels.

A number of possible fates await Ca^{2+} which enters the cytosol. Since Ca^{2+} is purportedly a second messenger in a signal transduction cascade, we might expect that some of the Ca^{2+} which enters the cytosol during agonist stimulation would serve this function by binding to effectors which will then propagate the signal to produce the intended response. A number of Ca^{2+}-binding proteins have been identified which are believed to be involved in signal transduction, including troponin C, calmodulin, PKC, cytoskeletal elements (e.g., annexins), and many others whose precise functions are as yet unknown (for review see Villereal and Palfrey 1989; V. L. Smith et al. 1990). Unlike other cellular messengers, Ca^{2+} is not degraded and therefore must be removed from the cytosol in order to restore $[Ca^{2+}]_i$

to resting levels. Consequently, not all of the Ca^{2+} which enters the cytosol will reach these effector molecules, as a number of processes are at work to extrude or sequester Ca^{2+}.

Although free ionized Ca^{2+} concentration in the resting cell cytosol is maintained at around 0.1 μM, total cell Ca^{2+} concentrations have been estimated to be from 20 μM to 4 mM, depending on the cell type (reviewed by Carafoli 1987), indicating that most Ca^{2+} in resting cells is bound or sequestered. Many Ca^{2+}-binding proteins exist within cells, both soluble and membrane-associated; their ability to buffer Ca^{2+} is a function of their concentration and their affinity for Ca^{2+}. The most important soluble Ca^{2+} buffer in most eukaryotic cells is calmodulin. Calmodulin has four Ca^{2+} binding sites per molecule and is believed to mediate many of the signaling functions of Ca^{2+} in cells. With a concentration in nonmuscle cells estimated at around 30 μM, and a high affinity for Ca^{2+} (estimated at 0.4 μM when complexed with its cellular target proteins (V.L. Smith et al. 1990)), calmodulin may buffer substantial amounts of Ca^{2+}, particularly when $[Ca^{2+}]_i$ is elevated above resting levels. Most quantitative Ca^{2+} buffering is attributed to membrane-associated Ca^{2+}-binding proteins. In particular, the buffering capacity of Ca^{2+}-transporting proteins is not limited by their concentration since bound Ca^{2+} is translocated to the opposite side of a relatively impermeable membrane and the transporter protein is recycled to be available to bind more Ca^{2+} on the cytosolic side of the membrane.

8.1 Ca^{2+} Transport Proteins

At least five Ca^{2+}-transporting proteins are found in most mammalian cells. The plasma membrane contains a Ca^{2+}-ATPase and a Na^{2+}/Ca^{2+} (or Na^{+}/Ca^{2+} and K^{+} exchanger) which remove Ca^{2+} from the cytosol. Mitochondria have an electrophoretic Ca^{2+} uniporter for Ca^{2+} uptake and a distinct Na^{+}/Ca^{2+} exchanger which releases Ca^{2+} from the mitochondrial matrix. The sarcoplasmic (endoplasmic) reticulum has its own Ca^{2+}-ATPase which is distinct from the plasma membrane Ca^{2+} pump. These various transport systems have different affinities for Ca^{2+}, which has implications for their physiological functions. The plasma membrane (PM) and sarcoplasmic reticulum (SR) ATPases have high affinity (but low capacity) while the PM Na^{+}/Ca^{2+} exchanger and mitochrondrial transporters have low affinity but high capacity for Ca^{2+} transport.

The Ca^{2+}-ATPases of the PM and SR have been well characterized (for reviews see Schatzmann 1989; Carafoli et al. 1989; Inesi and Kirtley 1990). The PM Ca^{2+}-ATPase is apparently highly conserved in different tissues,

has a molecular weight of 140 kDa, and transports 1 molecule of Ca^{2+} for each molecule of ATP hydrolyzed. The SR Ca^{2+}-ATPase has a molecular mass of 110 kDa and transports two molecules of Ca^{2+} for each ATP hydrolyzed. Both pumps transport Ca^{2+} against a concentration gradient. They have a high Ca^{2+} affinity (0.1 – 1 μM) on the cytosolic (*cis*) side and a low Ca^{2+} affinity (2 – 10 mM) on the *trans* side (Schatzmann 1989). Both pumps require Mg^{2+} and have two ATP-binding sites with different affinities. A high affinity ATP site (1 μM K_d) is the hydrolytic site and a low affinity site (100 – 300 μM K_d) is a modulatory site which functions to increase the transport of Ca^{2+} when ATP is bound. Hydrophobicity analysis of primary amino acid sequences have suggested similar structural features of the pumps, including 8 – 10 transmembrane domains connected by large cytosolic regions which include both the C and N termini.

The most significant difference between these Ca^{2+} ATPases is in their modulation by calmodulin or other proteins. The PM pump binds calmodulin with high affinity (nM K_d) to its carboxy terminus and this binding increases the transporter's affinity for Ca^{2+} and ATP, 30fold and 100fold, respectively. The PM Ca^{2+}-ATPase has also been reported to be stimulated by phosphorylation by cAMP-dependent protein kinase (Caroni and Carafoli 1981) and PKC (Furukawa et al. 1989). The SR pump does not bind calmodulin, but the SR pump from cardiac, smooth, or slow twitch (but not fast twitch) skeletal muscle is associated with an acidic proteolipid, phospholamban. Phospholamban is a pentamer of identical 6-kDa subunits and is phosphorylated by cAMP-dependent protein kinase, Ca^{2+}-calmodulin-dependent kinase, and PKC. Phosphorylation of phospholamban is associated with stimulation of pump activity. This increased activity apparently results from dissociation of phospholamban from the Ca^{2+}-ATPase, indicating that phospholamban is an inhibitor of the pump (James et al. 1989). A 22-kDa protein which is distinct from phospholamban is associated with an intracellular platelet Ca^{2+}-ATPase and is phosphorylated by cAMP-dependent protein kinase and Ca^{2+}-calmodulin-dependent protein kinase, resulting in enhanced pump activity (Dean 1989). Endoplasmic reticulum Ca^{2+}-ATPases are less well characterized, but are generally believed to be analogous to the SR Ca^{2+} pumps.

The Na^+/Ca^{2+} exchanger of the PM has been studied predominantly utilizing heart and nervous tissue, in which it is found in high concentrations. In heart, the exchanger is believed to transport 3 Na^+ for 1 Ca^{2+}, while in rod outer segment it has recently been recognized to transport 4 Na^+ for 1 Ca^{2+} and 1 K^+ (Lagnado and McNaughton 1990). In either case, the exchanger is electrogenic, transporting one positive charge for each exchange cycle. The exchanger is reversible, capable of either removing Ca^{2+} from the cytosol or increasing Ca^{2+} entry, depending on the

ionic gradients and membrane potential. Its Ca^{2+} affinity, measured in isolated PM vesicles, is remarkably low (2–5 μ*M*), suggesting that its role in Ca^{2+} removal is restricted to situations where $[Ca^{2+}]_i$ is greatly elevated over resting levels, or alternatively, that the measured Ca^{2+} affinity does not reflect the true Ca^{2+} affinity of the exchanger in vivo (Carafoli 1987).

Mitochondria have long been known to be capable of sequestering Ca^{2+}. Ca^{2+} uptake into mitochondria is attributed to a uniporter which transports Ca^{2+} down its electrochemical gradient into the mitochondrial matrix (reviewed by Gunter and Pfeiffer 1990). Ca^{2+} uptake is cooperative (approximately second order) and is inhibited by ruthenium red and lanthanides. The affinity of this transporter for Ca^{2+} is low (K_d approx. 10 μ*M*) and the V_{max} (>1 μ*M*) an order of magnitude slower than that of SR (Carafoli 1987), suggesting that the role of mitochondria in buffering cytosolic Ca^{2+} is probably insignificant except in cases of injury or toxicity which introduce nonphysiological quantities of Ca^{2+} into the cytosol. Ca^{2+} release from the mitochondrial matrix occurs via a Na^+/Ca^{2+} exchange mechanism and by a Na^+-independent mechanism which has not been fully characterized (Gunter and Pfeiffer 1990).

In accord with their high Ca^{2+} affinities and relatively fast transport rates, the Ca^{2+} pumps of the plasma membrane and SR (endoplasmic reticulum) are believed to play an important role in regulating the rapid changes in $[Ca^{2+}]_i$ observed following agonist stimulation. As noted above, these transporters are likely to be activated by phosphorylation and/or calmodulin binding in response to stimulation and will therefore accelerate the decline in $[Ca^{2+}]_i$ back toward basal levels. These effects are particularly important in considering the mechanisms underlying agonist-induced oscillations in $[Ca^{2+}]_i$ which have now been observed in many cells types.

8.2 Ca^{2+} Reuptake and Ca^{2+} Oscillations

A number of mechanisms have been proposed to explain agonist-induced oscillations of $[Ca^{2+}]_i$. These have been reviewed by Berridge and Gallione (1988) and Berridge (1990). In nonexcitable cells, $[Ca^{2+}]_i$ oscillations involve a cyclic release of intracellular Ca^{2+} and are believed to be controlled by PI turnover. The mechanism favored by Berridge attempts to explain how $[Ca^{2+}]_i$ can oscillate under conditions where Ins(1,4,5)P_3 levels are stable. Such a mechanism could explain oscillations of $[Ca^{2+}]_i$ in response to a single injection of Ins(1,4,5)P_3 (Payne et al. 1988; Berridge 1988) or its nonmetabolizable analog, inositol trisphosphorothioate (Ins(1,4,5)P_3S_3) (Wakui et al. 1989; DeLisle et al. 1990). Berridge and

Gallione (1988) proposed a "two pool" model in which Ca^{2+} released from an Ins(1,4,5)P_3-sensitive intracellular store is taken up by an Ins(1,4,5)P_3-insensitive store; when the Ca^{2+} content of the Ins(1,4,5)P_3-insensitive store reaches some threshold level, the Ca^{2+} is released into the cytosol. A cycle of uptake and release of Ca^{2+} from the Ins(1,4,5)P_3-insensitive store would continue as long as the Ca^{2+} content of this store continues to reach the threshold necessary for release.

A key feature of this model is that the frequency of oscillations will depend on the rate of filling of the Ins(1,4,5)P_3-insensitive store. Ca^{2+} uptake into this store is presumably mediated by a Ca^{2+}-ATPase. Factors which would likely affect Ca^{2+} pumping would include the cytosolic Ca^{2+} concentration, the Ca^{2+} concentration within the store, and modulation of the pump (e.g., by phosphorylation). The rate of Ca^{2+} leak from the store would also affect the rate of filling of the store. Each of these factors will be changing with kinetics which are highly interrelated and dependent on the concentration of agonist. The frequency of agonist-induced oscillations of $[Ca^{2+}]_i$ have been shown to vary with the agonist concentration in many cases.

Goldbeter et al. (1990) have recently proposed a mathematical model to explain how $[Ca^{2+}]_i$ oscillations might arise based on the "two pool" hypothesis. The utility of such a model is limited because it greatly oversimplifies the complex interactions of the various Ca^{2+}-buffering components and makes assumptions which are almost certainly invalid in order to produce the desired oscillatory behavior. For example, Goldbeter et al. assume that buffering is linear with respect to Ca^{2+} concentration. This is highly unlikely given the variety of calcium-binding proteins with different affinities and different concentrations present in the cytosol, although we must concede that it would be impossible to devise a mathematical term which would take this into account. They also assume that Ca^{2+} entry exactly balances the release of Ca^{2+} from the Ins(1,4,5)P_3-sensitive store – both remaining constant, which is inconsistent with the measured changes in agonist-stimulated Ins(1,4,5)P_3 levels and contradicts a number of studies which have found that the rate of agonist-induced Ca^{2+} entry exceeds the rate of release of intracellular Ca^{2+}. The assumptions that Ca^{2+} leak into the cytosol remains constant while $[Ca^{2+}]_i$ oscillates and that Ca^{2+} extrusion varies linearly with $[Ca^{2+}]_i$ ignore the effects of Ca^{2+} oscillations on the driving force for Ca^{2+} entry and the complex modulation of pump activity as well as the potential recruitment of Na^+/Ca^{2+} exchange for Ca^{2+} extrusion. Despite these apparent flaws, the main features of the model may well approximate the actual mechanism.

The two pool model of agonist-induced $[Ca^{2+}]_i$ oscillations requires an Ins(1,4,5)P_3-insensitive Ca^{2+} store which releases Ca^{2+} by a Ca^{2+}-induced

Ca^{2+} release (CICR) mechanism. CICR is a phenomenon which has been most thoroughly characterized in cardiac myocytes where it is believed to be responsible for excitation-contraction coupling (for review see Fleischer and Inui 1989). In these cells, Ca^{2+} enters the cytosol through voltage-sensitive Ca^{2+} channels in the PM (sarcolemma) and triggers the release of Ca^{2+} from the SR. In skinned myocytes (in which the sarcolemma has been removed), release of intracellular Ca^{2+} (and consequent tension development) can be induced by addition of Ca^{2+} or caffeine. The Ca^{2+} release channel of heart and other muscle tissue is believed to be identical with a high molecular mass (360–400 kDa) ryanodine-binding protein. Ryanodine binding is biphasic; high affinity binding (K_d 5–10 nM) locks the channel in the open state, low affinity binding (K_d approx. 1 μM) blocks the channel. Recent evidence from isolated skeletal muscle SR (Nelson and Nelson 1990) suggests that the Ca^{2+} release channel has two Ca^{2+}-regulatory binding sites, a low affinity intraluminal site and a high affinity extraluminal site. Caffeine was found to decrease both the minimum intraluminal and minimum extraluminal Ca^{2+} concentration required to trigger Ca^{2+} release.

In smooth muscle cells, CICR may involve a separate intracellular Ca^{2+} store which is distinct from the Ca^{2+} stores released by histamine and norepinephrine. Matsumoto et al. (1990) examined the characteristics of histamine-, norepinephrine-, and caffeine-sensitive intracellular Ca^{2+} stores in primary cultures of rat vascular smooth muscle. They found that histamine and norepinephrine-sensitive Ca^{2+} stores were readily depleted by exposure to either of these agonists in Ca^{2+}-free medium, but the stores were replenished by a brief exposure to Ca^{2+}-containing medium. Histamine released all of the stored Ca^{2+} in the norepinephrine-sensitive stores, and norepinephrine released all of the histamine-sensitive stores. Neither of these agonists release the caffeine-sensitive stores and caffeine does not release Ca^{2+} from the histamine and norepinephrine-sensitive stores. Furthermore, release and replenishment of the caffeine-sensitive stores was much slower than the histamine/norepinephrine-sensitive stores, leading the authors to speculate that the caffeine-sensitive stores may be located in the central part of the cell with relatively poor communication with the extracellular environment, while the histamine/norepinephrine-sensitive stores may be located in close proximity to the cell surface.

A number of recent reports have suggested that CICR may be operating in nonmuscle cells. Kuba (1980) recognized that caffeine-induced oscillations of $[Ca^{2+}]_i$ in bullfrog sympathetic neurons suggested that these cells have Ca^{2+} storage sites similar to the SR. Lipscombe et al. (1988) recently described a characteristic spatial pattern of $[Ca^{2+}]_i$ changes in sympathetic neurons elicited by caffeine. They found that caffeine-induced $[Ca^{2+}]_i$

elevations were primarily localized to the cell body where intracellular Ca^{2+} stores are believed to be located. Malgaroli et al. (1990) have found that spontaneous $[Ca^{2+}]_i$ oscillations in rat chromaffin cells may be attributed to CICR. Their evidence includes increased frequency of oscillations and recruitment of nonoscillating cells by treatment with caffeine and inhibition of oscillations with 10 μM ryanodine. They also found that agonists which generate Ins(1,4,5)P_3 (bradykinin (BK) and histamine) also increased frequency of spontaneous oscillations and recruited nonoscillating cells, but unlike caffeine, BK and histamine effects were blocked by neomycin and unaffected by ryanodine. These results suggest that CICR can occur in rat chromaffin cells by a mechanism which is not strictly dependent on Ins(1,4,5)P_3-induced Ca^{2+} release. Caffeine-stimulated release of intracellular Ca^{2+} has also been demonstrated in bovine chromaffin cells (Burgoyne et al. 1989; Cheek et al. 1990).

Reports of CICR in nonexcitable cells have also been emerging. High affinity ryanodine-binding sites have been demonstrated in endoplasmic reticulum of rat liver (Shoshan-Barmatz 1990). A 360-kDa protein was found to cross-react with antibodies against the ryanodine receptor of rabbit SR and ^{3}H-ryanodine binding was measured, indicating a single binding site with a K_d of 8 nM. Ryanodine binding was inhibited by caffeine in a dose-dependent manner (K_i 3 mM). Shoshan-Barmatz also demonstrated that, at concentrations below 10 μM, ryanodine stimulated Ca^{2+} efflux from liver microsomes loaded with $^{45}Ca^{2+}$, while at higher concentrations it blocked the Ca^{2+} efflux induced by EGTA or low concentrations of ryanodine.

Marty and Tan (1989) found that microinjection of Ca^{2+} into rat lacrimal gland cells produced a regenerative $[Ca^{2+}]_i$ transient which was inhibited by ruthenium red, a known blocker of CICR in SR. Osipchuk et al. (1990) demonstrated that caffeine-enhanced $[Ca^{2+}]_i$ oscillations induced by perfusion of single rat pancreatic acinar cells with Ins(1,4,5)P_3 (caffeine alone was ineffective in eliciting $[Ca^{2+}]_i$ oscillations). Infusion of Ca^{2+} into these cells elicited transient $[Ca^{2+}]_i$ spikes similar to those elicited by Ins(1,4,5)P_3. Based on the recordings of fura2 fluorescence versus measurement of Ca^{2+}-activated Cl^- conductance changes, they concluded that CICR plays a role in the release of Ca^{2+} from stores located close to the plasma membrane. Wakui and Petersen (1990) recently followed up their earlier study by demonstrating that perfusion of single pancreatic acinar cells with caffeine produced transient $[Ca^{2+}]_i$ spikes when EGTA was not present in the pipette-filling solution. This response as well as the $[Ca^{2+}]_i$ spikes elicited by acetylcholine, Ins(1,4,5)P_3S_3, and Ca^{2+} were inhibited by externally applied ionomycin. Intracellular Ca^{2+} perfusion also inhibited caffeine and Ins(1,4,5)P_3S_3-evoked $[Ca^{2+}]_i$

spikes, suggesting a negative feedback effect of Ca^{2+} on the CICR mechanism.

The existence of distinct Ins(1,4,5)P_3-sensitive and insensitive intracellular Ca^{2+} stores in pancreatic acinar cells has been suggested by Thévenrod et al. (1989). Using permeabilized pancreatic acinar cells and endoplasmic reticulum vesicles isolated from these cells, they found that ATP-dependent Ca^{2+} uptake into an Ins(1,4,5)P_3-sensitive store is mediated by a MgATP-dependent Ca^{2+}/H^+ exchange mechanism. An Ins(1,4,5)P_3-insensitive store takes up Ca^{2+} by a vanadate-sensitive Ca^{2+}-ATPase which is capable of buffering cytosolic [Ca^{2+}] at near resting levels ($K_d = 7 \times 10^{-7} M$). The Ins(1,4,5)P_3-sensitive store is filled at higher $[Ca^{2+}]_i$ ($K_d = 2 \times 10^{-5} M$).

Burgoyne et al. (1989) have shown that bovine adrenal chromaffincells contain two Ca^{2+} ATPase-like proteins with distinct subcellular distributions. Using two different monoclonal antibodies raised against the skeletal muscle Ca^{2+}-ATPase, they identified two distinct proteins in chromaffin cells which were differentially recognized. A 100-kDa proteins was distributed diffusely throughout the cytosol and a 140-kDa protein had a perinuclear distribution. Fura2 imaging also revealed characteristic spatial patterns of $[Ca^{2+}]_i$ elevation in response to A_{II} or caffeine. $[Ca^{2+}]_i$ elevations elicited by A_{II} (which activates PI turnover in these cells) tended to originate in a restricted area between the nucleus and the plasma membrane. In contrast, caffeine produced uniform $[Ca^{2+}]_i$ elevations throughout the cytosol. Burgoyne et al. concluded that distinct Ins(1,4,5)P_3-sensitive and caffeine-sensitive intracellular Ca^{2+} stores exist with characteristic intracellular distributions. They further speculate that the caffeine-sensitive stores may be identical to the calciosomes described by Volpe et al. (1988), which have been found to be distributed throughout the cytosol and to be recognized by antibodies against an SR Ca^{2+}-ATPase which also recognizes a 100-kDa protein in liver.

In a recent report on calcium oscillations in fibroblasts, it was suggested that fibroblasts have a internal store of Ca^{2+} which can be released by an increase in cytosolic Ca^{2+} (Harootunian et al. 1991). It was shown that elevation of cytosolic Ca^{2+} by either wounding the cell membrane or by the photolysed release of caged Ca^{2+} could cause a release of internal Ca^{2+} and induce Ca^{2+} oscillations. However, this Ca^{2+}-induced Ca^{2+} release is clearly different from that seen in excitable tissues because neither Ca^{2+} release under basal conditions or Ca^{2+} oscillations were sensitive to caffeine or ryanodine. Also, the Ca^{2+}-induced oscillations differed from those seen in excitable cells in that mitogen stimulation of cells was necessary to see Ca^{2+} oscillations induced by Ca^{2+} injection.

The picture which seems to emerge from all of these studies is that both excitable and nonexcitable cells may have at least two nonmitochondrial

intracellular Ca^{2+} stores: an $Ins(1,4,5)P_3$-sensitive store and a caffeine- or Ca^{2+}-sensitive store, with a contribution from Ca^{2+} entry. These stores may interact to produce complex agonist-induced $[Ca^{2+}]_i$ responses, including oscillations of $[Ca^{2+}]_i$. These stores will also contribute to cytosolic Ca^{2+} buffering and may, therefore, play an important role in intracellular Ca^{2+} homeostasis (Cheek et al. 1990).

Another aspect of intracellular $[Ca^{2+}]$ regulation which is emerging concerns the role of Na^+ in $[Ca^{2+}]_i$ homeostasis in nonexcitable cells. The importance of Na^+/Ca^{2+} exchange for Ca^{2+} extrusion during relaxation of cardiac muscle and adaptation to light in rod cells has been recognized for some time. In nonexcitable cells, most extrusion of Ca^{2+} is probably accomplished by the plasma membrane Ca^{2+}-ATPase. Whether Na^+/Ca^{2+} exchange contributes to Ca^{2+} removal or, conversely, to Ca^{2+} entry in nonexcitable cells remains uncertain. A calculation based on the electrochemical equilibria for Na^+ and Ca^{2+} ($[Na^+]_i = 15$ m*M*, $[Na^+]_o = 150$ m*M*, $[Ca^{2+}]_0 = 1.8$ m*M*, $E_m = -75$ mV) reveals that a Na^+/Ca^{2+} exchange with a stoichiometry of 3 Na^+ : 1 Ca^{2+} will reverse at an intracellular $[Ca^{2+}]$ of 100 n*M* (Villereal 1986). If we assume that these are the conditions expected in a typical resting cell and that this resting $[Ca^{2+}]_i$ is achieved by the Ca^{2+}-ATPases of the PM and SR, the Na^+/Ca^{2+} exchange will produce no net Ca^{2+} or Na^+ transport in the resting cell. Given its relatively low affinity for intracellular Ca^{2+} (Carafoli 1987), significant Ca^{2+} extrusion by the exchanger at $[Ca^{2+}]_i$ below 1 μ*M* is doubtful. This has led some investigators to speculate that the Na^+/Ca^{2+} exchanger may function to increase Ca^{2+} entry into cells (Snowdowne and Borle 1985). More recently, mechanisms other than Na^+/Ca^{2+} exchange have been proposed to account for the effects of lowering extracellular Na^+ on $[Ca^{2+}]_i$ (Muallem et al. 1988c; J.B. Smith et al. 1989a; Negulescu and Machen 1990).

9 Relationship Between Other Mitogen-Stimulated Events and the Mitogen-Induced Ca^{2+} Response

Any discussion of the Ca^{2+} response in the growth factor signal transduction process should include at least a brief account of the role of Ca^{2+} in later mitogen-induced events. It is well accepted that the effects of Ca^{2+} elevation on cellular metabolism are mediated via a wide range of Ca^{2+}-binding proteins such as calmodulin and protein kinase C. Since the role of these Ca^{2+}-binding proteins has been recently reviewed (Villereal and Palfrey 1989), we will not go into detail about these mediators of the

Ca^{2+} signal. Instead, we will spend a short amount of time discussing the evidence that Ca^{2+}, acting through these mediators, helps regulate some important events in the mitogen-signaling process.

9.1 Role of Ca^{2+} in Regulating the Na^+/H^+ Exchanger

Early evidence from our laboratory (Villereal 1981; Muldoon et al. 1987; Villereal 1988) suggested that the elevation of $[Ca^{2+}]_i$, induced by mitogen stimulation of cultured human fibroblasts, was important in the activation of the Na^+/H^+ exchanger, which occurs within a minute of mitogen activation. Other evidence from our laboratory suggested that this effect was mediated by calmodulin (Owen and Villereal 1982). The role of Ca^{2+} in the activation of Na^+/H^+ exchange is now supported by a number of studies in different cells types (Rosoff and Terres 1986; Ober and Pardee 1987; Hendey et al. 1989; Gillies et al. 1989; Hesketh et al. 1985; Shen 1989; Manganel and Turner 1990). Evidence from other laboratories implicated PKC in this activation process (Moolenaar et al. 1984a) and some of these reports questioned the involvement of Ca^{2+} as a regulator of this transporter. However, recent studies from our laboratory on the activation process in a variety of cell types now indicate that the involvement of either Ca^{2+} or PKC depends on the cell type investigated. There are a number of cells in which PKC is the principal regulator of the Na^+/H^+ exchanger, while in other cell types it appears to play no role in the activation process (Etscheid et al. 1990).

9.2 Role of the Mitogen-Induced Ca^{2+} Signal in the Phosphorylation of Elongation Factor 2

Early studies in our laboratory (Muldoon et al. 1987) identified a 100-kDa phosphoprotein which was phosphorylated in response to mitogen stimulation of human fibroblasts. This protein could also be phosphorylated in response to addition of Ca^{2+} ionophores, implicating a Ca^{2+} regulated kinase system in the phosphorylation of this substrate. Concurrent studies by Palfrey and Nairn (Palfrey 1983; Nairn et al. 1985) had identified a 100-kDa substrate for a newly identified calmodulin-dependent kinase (CaM kinase III) and had generated an antibody to this protein. The 100-kDa substrate and CaM kinase III exhibited a unique relationship to each other. CaM kinase III was the only known kinase to phosphorylate the 100-kDa protein and the 100-kDa protein was the only known substrate of CaM kinase III. To determine whether the 100-kDa protein phosphory-

lated in a Ca^{2+}-dependent manner in our fibroblasts was the same as identified in other tissues as a substrate of CaM kinase III, we performed immunoprecipitation studies in cells labeled to isotopic equilibrium with ^{32}P. The antibody to the 100-kDa substrate of CaM kinase III was found to immunoprecipitate a 100-kDa protein from mitogen-reglated fibroblasts. Although some phosphorylated protein could be immunoprecipitated from quiescent cells, the number of counts incorporated into this protein was increased by fivefold with mitogen stimulation.

Although initial studies did not identify this protein beyond its molecular weight, subsequent studies (Nairn and Palfrey 1987) identified the protein as elongation factor 2. While elongation factor 2 is clearly important in the regulation of translation of protein, the exact role of the phosphorylation of elongation factor 2 by CaM kinase III is not presently known. It is interesting to note that the phosphorylation of elongation factor 2 is very transient in nature. The time course of the phosphorylation follows very closely the time course of the transient peak of the Ca^{2+} response. It is not clear at present what advantage a transient phosphorylation of elongation factor 2 would give to mitogen-stimulated fibroblasts.

9.3 Role of Ca^{2+} in Signaling Gene Transcriptional Events

One important aspect of mitogen stimulation of fibroblasts is the induction of mRNA for important genes which occurs rapidly (within 10–30 min) after mitogen addition. Much work has been done in trying to describe the mechanism by which the signal is transmitted from the surface receptor to the nucleus. There are several pieces of evidence which suggest that Ca^{2+} may be involved in this process. For example, in Balb/c 3T3 cells EGF is known to induce the mRNA for c-*fos* and c-*myc* (Ran et al. 1986). Since EGF is known to give a Ca^{2+} signal, these investigators determined whether an artificial elevation of $[Ca^{2+}]_i$ by calcium ionophores would induce the c-*fos* and c-*myc* mRNAs. They found that A23187, when given with an agent that would elevate cAMP, would give a significant increase in the induction of these two messages. In a similar study in our laboratory, we investigated whether the EGF-induced rise in message level for the EGR-1 gene (Sukhatme et al. 1987; Sukhatme et al. 1988) was dependent on the presence of external Ca^{2+}. We had previously shown that the EGF-induced Ca^{2+} response was dependent on the presence of external Ca^{2+}. We then demonstrated that removal of external Ca^{2+} would effectively block the EGF induction of the EGR message levels (Jamieson et al. 1989).

In a recent study in Rat-1 cells (Lenormand et al. 1990), a construct containing AP-1-like elements was used to assess the role of the second messengers PKC and Ca^{2+} on the regulation of chloramphenicol acetyltransferase (CAT) expression. It was observed that endothelin, which activates PKC and elevates $[Ca^{2+}]_i$ in Rat-1 cells, was effective in stimulating CAT expression. However, neither the addition of 12-*O*-tetradecanoylphorbo-13-acetate (TPA), to activate PKC, or thapsigargin, to elevate intracellular Ca^{2+}, was sufficient to stimulate CAT expression. However, when combined, TPA and thapsigargin produced a dramatic synergistic stimulation of CAT expression. These data suggest that Ca^{2+} may play a role in regulating genes which are under the control of AP-1-like elements.

9.4 Link Between the *ras* Oncogene and Ca^{2+} Regulation

In recent years several articles have been published which describe the interactions of the *ras* oncogene with the Ca^{2+} signal transduction process. Polverino et al. (1990) describe the effect of transforming NIH-3T3 cells with a *ras* oncogene on the bombesin stimulation of a Ca^{2+} response. They report a complete inhibition of the ability of bombesin to release intracellular Ca^{2+} as well as an inhibition of PDGF or bombesin stimulation of Ca^{2+} influx. The inhibition of the bombesin signal is observed to be accompanied by a decrease in the number of bombesin receptors. The authors conclude that one of the effects of *ras* expression is to inhibit the mitogen-stimulated Ca^{2+} influx by a mechanism which involves PKC. A similar inhibition of the bombesin-stimulated Ca^{2+} response is reported by another group for *ras*-transformed NIH-3T3 cells (Oberhuber et al. 1991). However, these authors conclude that the inhibition by *ras* expression is the result of an inhibition of the Ins(1,4,5)P_3-sensitive Ca^{2+} channels or by interference with the translocation of Ca^{2+} between intracellular Ca^{2+} pools.

In another recent paper (Lang et al. 1991), it was reported that the expression of *ras* in NIH-3T3 cells changes the bradykinin-stimulated Ca^{2+} response from a simple transient to one which produces oscillation in intracellular calcium. The authors also observed that in *ras*-minus cells loaded with GTP[S], bradykinin would also induce Ca^{2+} oscillations.

Finally, a report by Rane (1991) suggests that the expression of *ras* in fibroblasts results in the presence of a Ca^{2+}activated K^+ current which is missing in nontransformed cells. These authors suggest that the mechanism of *ras* activation of cells may have something to do with a change in the expression of ion channels present in the plasma membrane.

10 Ca^{2+} Involvement in the Regulation of the Cell Cycle

Much of our discussion to this point has centered on the Ca^{2+} response to the addition of growth factors and the role that this Ca^{2+} signal plays in the regulation of the G_0/G_1 transition in proliferating cells. The main reason for this focus in our discussion is that the overwhelming majority of the studies of Ca^{2+} and proliferation have dealt with the role of Ca^{2+} early in the interphase portion of the cell cycle. The exception has been those investigators who have worked on understanding the regulation of the cell cycle in oocytes, eggs, and embryos. There are a considerable number of studies in these systems which argue very strongly for the regulation of other aspects of the cell cycle by changes in $[Ca^{2+}]_i$. This represents a different kind of control mechanism than the one we have discussed so far. The regulation of Ca^{2+} and cell proliferation by growth factors represents an external form of control of the cell cycle, whereas the mechanisms we are about to discuss represent the means by which the cell controls the timing of internal events in the cell cycle once the cycle has been initiated by external factors.

10.1 Cell Cycle Control Points

A discussion of the role of Ca^{2+} in the control of internal events in the cell cycle should begin with a brief discussion of what is currently known about the points in the cell cycle where regulation occurs. Since much of what we know about this area comes from work with cell cycle mutants in yeast (see Lee and Nurse 1988 for review), we will briefly summarize some of what has been learned from that system. For a number of years investigators have studied cell cycle mutants of yeast to learn what proteins are involved in the regulation of the cell cycle. By selecting mutants which either divide at too large or too small a size investigators were able to select useful mutants which could then be used to clone cell division cycle (CDC) genes which are important for the regulation of the cell cycle. From these types of studies there has emerged a good understanding of the control points in the regulation of the yeast cell cycle. There are three such points that can be readily identified in yeast, START, mitosis ENTRY and mitosis EXIT. The START control point is just prior to transition from G_1 into S phase. Mitosis ENTRY is a point in G_2 just prior to mitosis onset. Mitosis EXIT is a point in M phase after chromosome segregation which is probably at the metaphase-anaphase transition. Studies in the fission yeast *Schizosaccharomyces pombe* and the budding yeast *Saccharomyces cerevisiae* have identified a particular gene CDC2 in *Saccharomyces*

pombe (CDC 28 is the equivalent gene in *Saccharomyces cerevisiae*) which appears to play a key role in the regulation of two important transition points in the cell cycle, START and ENTRY. Although these genetic studies identified the genes which are important in the regulation of the cell cycle, it was studies that approached the problem from a different direction which identified the functional importance of the gene products of the CDC2 gene. Biochemical studies had been going on for a number of years to identify the protein important in regulating mitosis in eggs and oocytes. An activity called maturation promotion factor (MPF) had been purified from mitotic oocytes which could induce resting oocytes to precociously mature when microinjected (Masui and Markert 1971; L.D. Smith and Ecker 1971). Recent biochemical studies (Gautier et al. 1988; Gautier et al. 1990) have identified the subunits of MPF to be the gene product of the CDC2 gene and cyclin, a protein which is known to be synthesized cyclically during the cell cycle.

10.2 Evidence for Ca^{2+} Involvement at Cell Cycle Control Points in Eggs

Further studies of the gene product of CDC2 identified it as a p34 protein which has serine/threonine kinase activity (Lee and Nurse 1987) and could itself be phosphorylated (Gautier et al. 1989). It is observed to be phosphorylated on both threonines and on tyrosines. The phosphorylation of this protein turns off its kinase activity. Since the p34 kinase is phosphorylated, an obvious question which arises is whether some second messenger-stimulated kinases can regulate the activity of p34 kinase and in particular whether p34 is regulated by a change in Ca^{2+} activity. In sea urchin eggs, it has been demonstrated that treatment of eggs with Ca^{2+} ionophore can induce the phosphorylation of p34 which mimics the phosphorylation of this protein observed with fertilization (Patel et al. 1989a, b). Since the fertilization of sea urchin eggs allows the immediate movement past START, these data suggest that Ca^{2+} is important in the role of p34 in regulating the movement of eggs past this important control point in the cell cycle.

Ca^{2+} also appears to play an important role at mitosis ENTRY. Cyclin, which is an important component of MPF, also appears to be phosphorylated in a Ca^{2+}-dependent manner. It appears that cyclin is constitutively phosphorylated following its synthesis, but in addition there is a burst of phosphorylation that occurs just prior to mitosis onset. Patel et al. (1989a, b) have observed that microinjection of calcium chelators will block both mitosis entry and the pulse of cyclin phosphorylation, implying that cyclin is either directly or indirectly phosphorylated in response to Ca^{2+}.

10.3 Changes in $[Ca^{2+}]_i$ During the Cell Cycle in Eggs and Embryos

Since experiments in which Ca^{2+} chelators are injected into cells argue for the involvement of Ca^{2+} at several critical cell cycle control points, it is important to discuss whether elevations in $[Ca^{2+}]_i$ occur at these particular times in the cell cycle. We have already discussed evidence that, soon after fertilization of eggs, a dramatic elevation in intracellular Ca^{2+} occurs (Steinhardt et al. 1977). Fertilization of eggs induces cell cycle progression (START) with a rapid (30 min) entrance into S phase. The elevation of internal Ca^{2+} can be obtained in Ca^{2+}-free medium, which points to the mobilization of intracellular Ca^{2+} stores as the mechanism for elevation of $[Ca^{2+}]_i$ (Chambers 1980; Chambers and Angeloni 1981; Schmidt et al. 1982; Crossley et al. 1988). Since artificial elevation of $[Ca^{2+}]_i$ by ionophores or by $Ins(1,4,5)P_3$ injection causes the cell cycle to resume (Twigg et al. 1988), this indicates that a rise in $[Ca^{2+}]_i$ is fundamental in the START signal.

There also appear to be increases in $[Ca^{2+}]_i$ associated with the other control points in the cell cycle. These signals are of particular interest because they occur in the natural progression of the cell cycle and are not a rapid response to the addition of an external agent as was seen for the fertilization spike. A small increase in $[Ca^{2+}]_i$ can be measured just minutes before mitosis ENTRY. Although this is normally a small signal a more dramatic increase at this time can be seen in polyspermic eggs (Whitaker and Patel 1990). It is possible that the difficulty in observing the Ca^{2+} spike at mitosis ENTRY may be the result of the Ca^{2+} change being localized to a small area and therefore being difficult to record on a cellular basis. Ca^{2+} spikes have also been observed at mitosis EXIT in sea urchin embryos (Poenie et al. 1985). It has also been observed that the presence of calcium chelators will arrest cells in mitosis, suggesting that the Ca^{2+} spike at mitosis EXIT may be necessary for the cells to complete the mitotic process.

10.4 Role of Ca^{2+} in the Regulation of Cell Cycle in Mammalian Somatic Cells

Although there is clear evidence for the role of Ca^{2+} in regulating the cell cycle at specific control points in eggs, oocytes and embryos, the picture is less well defined for somatic mammalian cells. To begin with, the control points in mammalian somatic cells are not as well defined as they are in yeast and eggs. Although there is substantial evidence for a restriction point in fibroblasts which occurs at approximately the same time in the cell

cycle as START, the story is not clear concerning what protein regulates the movement of cells past START. The best argument that control of the cell cycle in mammalian somatic cells is similar to that defined in yeast is that proteins homologous to the ones coded for by the yeast CDC genes are also found in mammalian cells. These mammalian homologs are thought to be functionally equivalent to their yeast counterparts (Lee and Nurse 1987). The evidence for Ca^{2+} involvement in regulating the cell cycle at these transition points in mammalian somatic cells is less well developed than in the egg and oocyte system. Some cell cycle spikes in Ca^{2+} have been observed in mammalian somatic cells (Poenie et al. 1985; Poenie et al. 1986). The most frequent observation is a Ca^{2+} transient at mitosis EXIT. However, a comparative study of Ca^{2+} changes at mitosis EXIT in different fibroblasts indicates that some cells show the transient signal and some do not. Whether the inability to see a Ca^{2+} transient at mitosis EXIT means that this signal is not necessary or merely that a technical problem in measuring local changes in Ca^{2+} exists remains to be seen. Clearly, an investigation of the Ca^{2+} signals around START and mitosis ENTRY need to be performed in mammalian somatic cells.

11 Summary

There is a substantial amount of information which has been obtained concerning the effects of growth factors on $[Ca^{2+}]_i$ in proliferating cells. A number of different mitogens are known to induce elevations in $[Ca^{2+}]_i$ and some characterization of the Ca^{2+} response to different classes of mitogens has been obtained. In addition, much is known about whether the Ca^{2+} response to a particular growth factor occurs as the result of an influx of external Ca^{2+} or a mobilization of internal Ca^{2+} stores. In addition, a considerable amount of information is available on the mechanism by which the Ins(1,4,5)P_3-sensitive internal Ca^{2+} store takes up and releases Ca^{2+}. However, there is still a large deficiency in our information concerning other Ca^{2+} stores in proliferating cells as well as in our knowledge of the mechanisms for regulating Ca^{2+} entry pathways. Much more data addressing these issues exists for other types of agonist-stimulated cells, and we have discussed much of it in this review article. While the wealth of data in nonproliferating cells provides some indications of what mechanisms might be involved in the growth factor-induced changes in $[Ca^{2+}]_i$, it is clear that much work must be done in proliferating cells to fully understand how external factors such as growth factors control $[Ca^{2+}]_i$. In addition, much work remains to be done in identi-

fying the mechanisms for the internal control of $[Ca^{2+}]_i$ as cells move through the cell cycle and in identifying the role that these changes in $[Ca^{2+}]_i$ may play throughout the cell cycle.

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Rev. Physiol. Biochem. Pharmacol., Vol. 119
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Serine/Threonine Kinases in the Propagation of the Early Mitogenic Response

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1 Introduction

In general, growth factors induce mitogenesis either through G proteins or tyrosine kinases. The first pathway involves the activation of receptor-coupled specific heterotrimeric G proteins through the binding of GTP and the release of the α subunit from the complex. In most cases, the α subunit-GTP complex acts through effector proteins to generate intracellular signals (Bourne et al. 1990). The second pathway is triggered by ligand activation either of receptor tyrosine kinase or tyrosine kinases associated with the receptor. These enzymes then phosphorylate specific target proteins modulating their effects (Ullrich and Schlessinger 1990; Cantley et al. 1991). A common feature of both pathways is the propagation of intracellular signals through serine/threonine phosphorylation (Cantley et al. 1991). It is the intent of this article to present a broad, general outline of those kinases implicated in this response. Figure 1 illustrates the discussed serine/threonine kinases, their interactions with one another and their role in the mitogenic response. For more information regarding individual kinases readers are referred to detailed review articles.

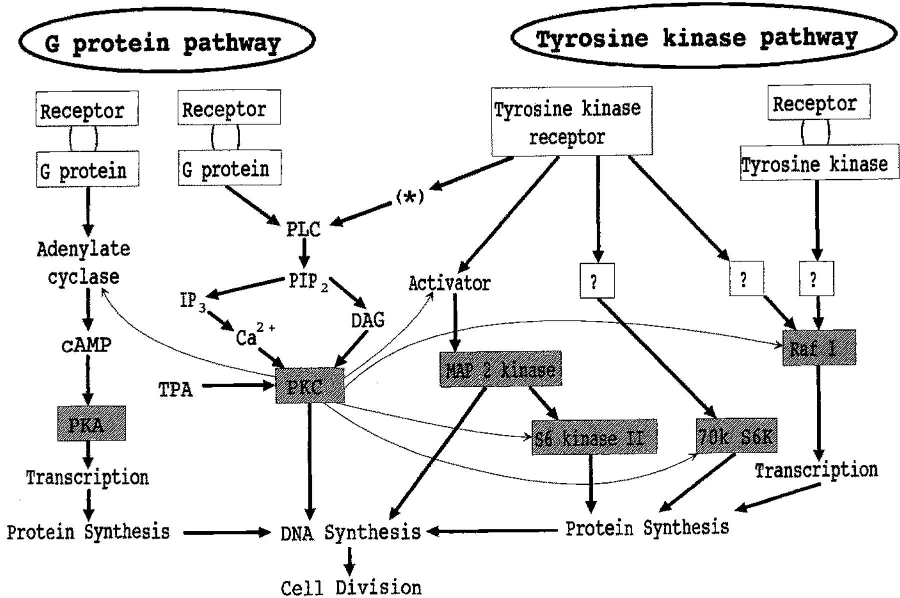

Fig. 1. Possible pathways involved in the mitogenic response. (), indicates two proteins which directly interact with one another; (*), only some tyrosine kinase receptors use this pathway; ?, mediator(s) unknown; →, indicates either indirect or direct signal transduction; *thin curved arrows* indicate a role for protein kinase C in the activation of other molecules

2 Cyclic AMP-Dependent Protein Kinase

Although the role of the cyclic AMP-(cAMP)-dependent protein kinase (PKA) in cell growth control has been a controversial subject (for a review see Dumont et al. 1989), a strong case can now be made for PKA in the mitogenic response (see below). PKA is a holoenzyme complex of two regulatory (R) subunits and two catalytic (C) subunits bound together in the absence of cAMP (Walsh et al., 1968). The isoforms of the holoenzyme were initially classified as type I or type II on the basis of their elution from a diethylaminoethanol (DEAE) column (Corbin et al. 1975). This difference was later shown to be due to different R subunits (Zoller et al. 1979). In addition, a number of genes have been cloned for R subunits as well as C subunits (for a review see Beebe and Corbin 1986). PKA is activated by hormonal stimulation of adenylate cyclase, mediated by the α subunit of a heterotrimeric G protein complex, leading to increased levels of intracellular cAMP (Gilman 1987; Simon et al. 1991). The heterotrimeric complex is made up of the subunits α, β and γ. In the case of the adenylate cyclase system, the α subunit has been designated the stimulatory regulator and is referred to as α_s. The α_s subunit includes the GTPase activity and the structural determinants for coupling specific receptors with the appropriate effector molecules. The β and γ subunits allow the α subunit to interact with receptors and increase the rate of GTP exchange with GDP but appear to play no role in the discrimination between different subsets of receptors. A growth factor acting through the adenylate cyclase pathway first binds to its receptor and promotes the released of GDP, allowing GTP to bind to the α_s subunit. The $\alpha_s \cdot \beta \cdot \gamma$ trimer dissociates from the receptor and then the GTP$\cdot \alpha_s$ subunit release from the $\beta\gamma$ complex is free to activate the effector molecule, adenylate cyclase. The interaction with adenylate cyclase is terminated by the α_s subunit-catalyzed hydrolysis of GTP. The GDP$\cdot \alpha_s$ then binds to the $\beta\gamma$ and the heterotrimer can again be reactivated by a second receptor molecule. Although the growth factor receptor complex is short-lived, existing for less than 1 s, it can generate one or more GTP-bound α_s chains which remain active for up to 10 s. This mechanism leads to amplification of the transducing signal and the accumulation of cAMP (Bourne et al. 1990). On binding of cAMP to the R subunit, the inactive holoenzyme dissociates into a dimeric R subunit and two active C subunits. The latter are then free to catalyze the phosphorylation of a number of specific protein substrates (Taylor 1989) containing the consensus sequence R-R-X-S/T-X (Kemp and Pearson 1990). PKA activation is reversed by the hydrolysis of cAMP by specific phosphodiesterases and the released dimeric R subunit is then free to bind to C subunits and neutralize their activity (Beavo 1988).

2.1 The R Subunits

The carboxy-terminal two-thirds of the type I and II R subunits display a high order of sequence conservation (for a review see Taylor 1989). This segment contains two tandem cAMP-binding domains which show high homology to the catabolite gene activator protein (CAP) in *Escherichia coli.* Based on the crystal structure of CAP, a model of the cAMP-binding domains has been constructed (Weber et al. 1987). Data from mutagenesis (Ji Buechler and Taylor 1991) and photoaffinity labeling (Adams et al. 1991) largely support the proposed framework for the folding of the R subunit. This model will be invaluable in generating protein crystals of the R subunit.

The amino-terminal third of the protein shows much less sequence homology between the different R subunits. The first 40–50 amino acid residues include the site responsible for dimerization of the R subunits. At approximately 90–100 amino acid residues from the amino-terminus of the protein there is a more conserved, proteolitically sensitive hinge region which is the site of interaction with the C subunit. The type II R subunits contain a phosphorylation site in this hinge region R-R-X-S-V, while the type I R subunits have a pseudophosphorylation site R-R-G-A/G-V/I as well as a high-affinity binding site for MgATP (Taylor et al. 1990). Corbin and colleagues (Corbin et al. 1978) first suggested that this hinge region inhibits the activity of the C subunit by binding to the active site of the kinase. Indeed, PKA was the first example of a kinase containing a pseudosubstrate sequence. A second and potent physiological inhibitor of the free C subunit is the heat-stable inhibitor commonly referred to as the protein kinase inhibitor (PKI) (Walsh et al. 1971). PKI is a 75-amino-acid-protein with a very high affinity for PKA, K_i 0.09 nM (Van Patten et al. 1991). As for the R subunit, inhibition has been shown to be mediated by a small segment of the protein containing the pseudosubstrate sequence R-R-N-A-I (Scott et al. 1985).

2.2 The C Subunit

The catalytic domains of all protein kinases are highly homologous (Hanks et al. 1988). Furthermore, the catalytic domain of the C subunit has been used as a model for other serine/threonine protein kinases, allowing a better understanding of the mechanisms of phosphate transfer and the generation of protein kinase family trees (Taylor et al. 1988; Hanks et al. 1988; Taylor 1989). The amino-terminal portion of the C subunit contains the ATP-binding site characterized by the motif G-X-G-X-X-G. This

motif is conserved in many nucleotide-binding proteins and, based on a model of the tyrosine kinase v-*src* (Sternberg and Taylor 1984), can be folded into an elbow around the nucleotide such that the first glycine residue, G_{50}, makes contact with the ribose moiety. Directly downstream of this sequence in all protein kinases in an invariant lysine residue, K_{72}, which appears to be directly involved in the phospho-transfer reaction (Kamps and Sefton 1986). Mutagenesis of this site to any other amino acid, including arginine, renders the kinase inactive (see Hanks et al. 1988).

The central portion of the C subunit appears to be involved in catalysis and substrate binding. An invariable aspartic acid residue in all protein kinases, D_{184} in PKA, takes part in the phospho-transfer reaction. Upstream of this residue is a glutamic acid, E_{170}, which is conserved in serine/threonine kinases having a requirement for basic residues in the substrate and which is replaced by a basic amino acid in kinases requiring acidic groups; thus this part of the sequence is probably involved in substrate interaction (Taylor 1989). A complete understanding of the molecular structure of the C subunit will require detailed crystallographic studies. Crystals for such studies have been produced for the apoenzyme (Sowadski et al. 1984) as well as a teriary complex made up of one molecule each of MgATP, an inhibitory peptide based on PKI, and the C subunit (Taylor 1989). It should also be noted that the high sequence homology of protein kinases means that the crystal structure of the C subunit will be invaluable for modeling other protein kinases.[1]

2.3 PKA as a Mitogenic Mediator

The role of PKA in growth promotion has been controversial (Boynton and Whitfield 1983), but it is now clear that it enhances or initiates cell proliferation in several cell types (for a review see Dumont et al. 1989). Thyrotropin (TSH), for example, regulates the synthesis and secretion of thyroid hormones in thyrocytes and also mediates cell growth in this tissue. Binding of TSH to its receptors leads to rapid, sustained elevation of cAMP, an effect that can be mimicked by agonists of adenylate cyclase such as cholera toxin and forskolin, as well as by cAMP analogs. The TSH effect is mediated through the TSH receptor-activated G_s protein as described above (Bourne et al. 1990). Very similar results are also elicicted by β-adrenergic agonists on parotid cells in vivo and in vitro (Tsang et al. 1980). Indeed, the effect of this signaling pathway on mitogenesis has

[1] After the completion of this article the crystal of the catalytic subunit of the cyclic AMP-dependent kinase was resolved C.

recently received a great deal of attention after mutant forms of G_s were found in human pituitary and thyroid tumors (Landis et al. 1989; Lyons et al. 1990). Mutations in either of two conserved amino acids impair G_s-GTPase activity and lead to a constitutively activated adenylate cyclase and a bypass of the normal growth factor requirement. These mutations have been denoted the *gsp* oncogene (Landis et al. 1989; Lyons et al. 1990).

In Swiss 3T3 cells, several agents induce an increase in intracellular cAMP level, including prostaglandin E_1, cholera toxin and forskolin. However, none of these agents alone is sufficient to induce DNA synthesis and cell growth. Other agents such as vasopressin will activate the protein kinase C (PKC) pathway (see below) but have no effect on cAMP level or DNA synthesis. However, any two agents from these two classes when combined are effective in initiating cell growth (Rozengurt 1986). Given more knowledge of specific substrates, these agonists will help to elucidate how these pathways interact to lead to cell growth.

2.4 Cellular Targets of PKA

The targets for PKA during mitogenesis remain obscure. It is known that PKA stimulates transcription of specific genes through a cAMP-responsive element (Montminy and Bilezekjian 1987). This element is activated by the binding of a protein of M_r 43000, the PKA-responsive element binding protein (CREB) which requires phosphorylation by PKA for transcriptional induction (Gonzalez and Montminy 1989). A role for PKA in iducing the transcription of specific genes is supported by the demonstration that transient transfection of a gene coding for PKI blocks the ability of cAMP to activate gene expression in COS cells (Grove et al. 1987). These results are compatible with the localization of the C subunit in the nucleus (Meinkoth et al. 1990).

In contrast, the R subunit type I, like the holoenzyme, is localized in the cytoplasm (Meinkoth et al. 1990; Adams et al. 1991). It has been suggested that the type I R subunit, apart from regulating the C subunit, serves as a "cytoplasmic anchor" keeping the C subunit near potential substrates (Meinkoth et al. 1990). Further elucidation of the role of PKA in cell growth control requires the identification of the substrates mediating its function.

3 Protein Kinase C

PKC was originally detected as a protease-activated serine/threonine kinase from rat brain (Takai et al. 1977; Inoue et al. 1977). Proteolytic cleav-

age and activation occur at one or two specific sites and result in the release of a catalytically active fragment of the kinase. It was subsequently shown that PKC activation is dependent on the presence of Ca^{2+} and cell membrane lipid-soluble factors. As a lipid-soluble factor, purified phosphatidylserine was the most efficient of the naturally occurring phospholipids tested. However, the observation that at low levels of Ca^{2+} crude lipid extracts from brain were still much more effective than phosphatidylserine alone led to the discovery of a neutral lipid, diacylglycerol (DAG), which activated PKC in a phosphatidylserine- and Ca^{2+}-dependent manner. Thus PKC activation was coupled to major signaling pathways involving the hydrolysis of phosphatidylinositols. Perhaps just as striking was the finding that PKC also proved to be the major cellular receptor for tumor-promoting phorbol esters acting through the same DAG binding site, implicating PKC in carcinogenesis and mitogenesis (for reviews see Nishizuka 1988; Jaken 1990).

It is now clear that growth factors stimulate PKC through activation of type C phospholipases (PLCs), which in turn hydrolyze inositol lipids, particularly phosphatidylinositol-4,5-bisphosphate (PIP_2) generating DAG and inositol-1,4,5-trisphosphate (IP_3) (Berridge and Irvine 1989). In the presence of phospholipids DAG leads to activation of PKC. In the case of the Ca^{2+}-dependent forms of PKC (see below) this effect is seen to be further augmented by IP_3, which provokes a rise in intracellular Ca^{2+} levels. PLCs are activated by both G protein-coupled receptors as well as several tyrosine kinase receptors (for a review see Meldrum et al. 1991). Given the analogy with the adenylate cyclase system described above, it is not surprising that PLC is activated via G proteins by such mitogens as bombesin, bradykinin, thrombin, and vasopressin. However, in the case of mitogens like epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), this effect appears to be mediated by direct phosphorylation of PLC (Wahl et al. 1988; Meisenhelder et al. 1989). In both cases phosphorylation is at tyrosine residues of a specific isoform of PLC, γ_1. Furthermore, it can be shown by coprecipitation studies that PLCγ_1 interacts directly with both receptors (Margolis et al. 1989; Meisenhelder et al. 1989). It should be noted that phosphatidylcholine also serves as a source of DAG, which may be important in long-term potentiation of the PKC signal (Pessin et al. 1990), and that other lipids can regulate the activity of PKC in vitro, including arachidonic acid and *cis*-unsaturated fatty acids (for a review see Bell and Burns 1991).

3.1 Different PKC Types

Several PKC enzymes have been purified from many eukaryotic sources and their genes cloned (for a review see Stabel and Parker 1991); we will

limit our discussion to the nine mammalian PKC types reported to date. These enzymes fall into two broad groups according to their dependence on Ca^{2+} for activation. The Ca^{2+}-dependent group includes PKC subtypes α, β_I, β_{II}, and γ, and the Ca^{2+}-independent group includes PKC subtypes δ, ε, ε', ζ, and η.

The PKC molecule can be divided into two domains, the amino-terminal regulatory domain and the carboxy-terminal catalytic domain. Within the regulatory domain is a conserved region containing a pseudosubstrate sequence (see below) and either one or two "zinc finger" motifs as found in nucleic acid binding and metalloproteins (Berg 1986). This region is involved in binding DAG and phorbol esters as well as containing the phospholipid interaction site. A second conserved region further downstream is exclusive to the regulatory domains of PKC types α, β_I, β_{II}, and γ and is therefore thought to confer the Ca^{2+}-dependent activation mechanism (Ono et al. 1988). The highly conserved carboxy-terminal half of the molecule contains the catalytic domain as well the substrate-binding site (Stabel and Parker 1991). In the phylogenetic tree of protein kinases, the PKC family is the second largest group after the tyrosine kinases (Hanks et al. 1988) and includes the PKA family. These kinases can be classified according to their mode of activation by either cyclic nucleotides or Ca^{2+}-phospholipids. As noted by Hanks et al. (1988), their activation through second messengers released at the cell surface may reflect a recent evolutionary divergence.

3.2 Site Specificity and the Pseudosubstrate Sequence

Synthetic peptides have been used to determine the PKC substrate-recognition determinants (House et al. 1987). The best substrates contain basic residues on each side of the target S/T residue with the following spacing R-X-X-S/T-X-R (Graff et al. 1989). The regulatory domains of all members of the PKC family contain such a consensus sequence, although an alanine replaces the serine/threonine residue (House and Kemp 1987). This pseudosubstrate site is thought to control kinase activity by interacting with the catalytic domain in the substrate-binding site. Binding of DAG or phorbol ester may in turn cause a conformational change that releases the pseudosubstrate site from the substrate-binding site, allowing specific substrates to bind (Parker et al. 1989). A number of studies support the argument that the pseudosubstrate sequence in the regulatory domain directly interacts with the substrate-binding site in the catalytic domain and maintains PKC in an inactive conformation in the absence of appropriate cofactors. First, a 29-amino-acid peptide corresponding to the putative

substrate-binding domain could activate PKC in the absence of phospholipid (House et al. 1989). Second, an antibody generated against the pseudosubstrate sequence when incubated with PKC in the absence of Ca^{2+} and phospholipids led to activation of the enzyme (Makowske and Rosen 1989). Finally, alteration of the pseudosubstrate sequence by site-directed mutagenesis significantly increased effector-independent PKC activity (Pears et al. 1990).

3.3 PKC as a Mitogenic Mediator

Two different approaches have been employed to evaluate the role of PKC in the mitogenic response. First, chronic activation of PKC in vivo by prolonged treatment of cells in culture with phorbol esters leads to downregulation of the enzyme, an effect mediated by proteolysis. The exact mechanism is unclear but the increased susceptibility of the active conformation of PKC to proteases in vitro suggests that this may simply reflect a passive response (see Stabel and Parker 1991). Exploiting this technique, Williams and coworkers (Coughlin et al. 1985) showed that downregulation of PKC had no effect on PDGF-induced DNA synthesis. Though a number of criticisms can be levied against this conclusion, it is supported by recent studies of Seuwen et al. (1990) who expressed the human M1 muscarinic acetylcholine (Hm1) receptors in Chinese hamster lung fibroblasts. When the receptor was activated by the muscarinic agonist carbachol, they observed a strong activation of PLC, and presumably PKC, as well as many of the other responses normally observed in early G_1, but no increased DNA synthesis. In contrast, Šuša et al. (1989) have shown that the ability of EGF to induce S6 phosphorylation (see below), protein synthesis and cell growth is attenuated following phorbol ester-induced downregulation of PKC in Swiss 3T3 cells. This suggests that PKC plays a positive role in cell growth, but this may depend on the cell type as well as the growth factor employed.

Another approach in testing the role of PKC in the mitogenic response has been to overexpress the PKC gene in mammalian cells. Such transformed cells exhibit altered morphology and increased growth rates following phorbol ester treatment (Housey et al. 1988; Krauss et al. 1989; Persons et al. 1988). In Swiss 3T3 cells, the overexpression of PKCα was accompanied by a decrease in both high- and low-affinity EGF receptors (Eldar et al. 1990). This decrease was attributable to lower levels of EGF receptor mRNA transcripts, suggesting that the expression of EGF receptors is regulated by PKCα. The authors point out that similar mechanisms may be involved in the initial stages of neoplasia and tumor promotion. It is

interesting to note that overexpression of PKCβ potentiates transformation by the H-*ras* oncogene (Hsiao et al. 1989) and that transformation modulates the expression of the different PKC subtypes in the same cell. For example, *ras* expression increases PKCα expression and decreases PKCε expression in Rat-6 fibroblasts (Borner et al. 1990). The relative amounts of the different PKC subtypes influence cellular growth and might explain why overexpression of PKCβ causes growth inhibition and tumor suppression in a colon cancer cell line (Choi et al. 1990).

3.4 Cellular Targets of PKC

As for PKA, little is known about the physiological substrates of PKC during the mitogenic response (for a review see Stabel and Parker 1991). One of the early candidates for a PKC substrate was a protein M_r 80000 subsequently referred to as the myristoylated, alanine-rich, protein C kinase substrate, or MARCKS (Stumpo et al. 1989). Its distribution coincides closely with that of PKC, thus making it a likely mediator of certain PKC functions. Rapid phosphorylation of MARCKS is detected in Swiss 3T3 cells treated with mitogens which exploit the PKC pathway (Rozengurt 1986). It has been shown recently that PKC-dependent phosphorylation of MARCKS displaces the protein from the plasma membrane and that dephosphorylation allows it to reassociate with membranes (Thelen et al. 1991). Furthermore, MARCKS is an actin-binding protein which can be colocalized with vinculin and talin at substrate-adherent focal contacts. Displacement of MARCKS from such focal contacts by PKC phosphorylation may be a prerequisite for rearrangement of the cytoskeleton (Thelen et al. 1991) and initiation of early mitogenic responses. Other possible PKC substrates include adenylate cyclase (Yoshimasa et al. 1987) and the inhibitor I-κB of the transcriptional activator NF-κB (Ghosh and Baltimore 1990). It should be noted that adenylate cyclase as a common substrate would allow crosstalk between the PKA- and PKC-dependent pathways. Finally, it is clear that PKC may operate indirectly through other kinases, including the protooncogene serine/threonine kinase Raf-1 (Siegel et al. 1990), the microtubule-associated protein 2 (MAP2) kinase (Rossomando et al. 1989) and the 70K S6 kinase (Ballou et al. 1988a; Šuša et al. 1989).

4 Raf-1 Kinase

Raf-1 is a serine/threonine kinase which is activated by a number of growth factors acting through tyrosine kinases (for a review see Li et al.

1991). The Raf-1 gene has been identified as the cellular counterpart or protooncogene of the oncogene v-*raf* from murine sarcoma virus 3611 (Bonner et al. 1986). The v-*raf* gene product is a constitutively activated serine/threonine kinase which in vitro is able to phosphorylate histone H5 and actin (Moelling et al. 1984). Two additional genes closely related to Raf-1, A-Raf and B-Raf, have recently been isolated (Beck et al. 1987; Ikawa et al. 1988). While Raf-1 expression is ubiquitous, A-Raf and B-Raf expression is restricted to urogenital and brain tissues, respectively (Storm et al. 1990). Of the three forms of Raf, Raf-1 has been the most studied so far.

4.1 Structure of Raf-1 Kinase

The Raf-1 gene product has a M_r 74000 and consists of two structural domains. The amino-terminal half of the molecule, which includes the regulatory domain, contains a phosphorylation site between amino acids 32 and 50 (Kovacina et al. 1990; App et al. 1991) and a cysteine-rich region with a zinc finger motif (Berg 1986) between amino acids 132 and 148. Between residues 256 and 268 is a region rich in serine and threonine residues which is conserved in all members of the Raf gene family (Heidecker et al. 1989). The carboxy-terminal half of the molecule, from amino acids 330 to 648, contains the catalytic domain and defines Raf-1 as a member of the serine/threonine kinases. Nevertheless, according to the phylogeny of protein kinase catalytic domains Raf-1, a few kinases related to Raf-1, and Mos lie very close to the larger group of tyrosine kinases (Hanks et al. 1988). The mechanism by which the regulatory part of Raf-1 controls kinase activation is not understood. It has been suggested that the zinc finger motif could be a ligand-binding site such as in PKC, but no ligand has as yet been identified for Raf-1 (Rapp et al. 1988). The importance of the regulatory domain has been made clear from the sequence of v-*raf* isolates and transforming Raf-1 recombinants obtained by genomic DNA transfection in murine fibroblasts. Constitutive activation of the kinase is achieved either by deletion of the amino-terminal half of the kinase, by fusion of an unrelated sequence to the full-length kinase or by disruption of the conserved serine/threonine-rich region common to Raf family members (Shimizu et al. 1985; Ishikawa et al. 1987, 1988; Mitsunobu 1989; Stanton et al. 1989; Heidecker et al. 1990).

4.2 Raf-1 Kinase as a Mitogenic Mediator

The role of Raf-1 in mitogenesis was first approached through a set of antibody microinjection studies carried out by Stacey and colleagues

(Mulcahy et al. 1985; Smith et al. 1986). These authors initially demonstrated that EGF- and PDGF-induced DNA synthesis of NIH 3T3 cells could be blocked by prior microinjection of an antibody directed against the proto-oncogene c-*ras* (Mulcahy et al. 1985). Similarly, they showed that microinjection of the anti-*ras* antibody reverted NIH 3T3 cells transformed either by a tyrosine kinease receptor oncogene, v-*fms*, or a receptor-coupled tyrosine kinase oncogene, v-*src*, to the normal phenotype (Smith et al. 1986). However, v-*raf*-transformed cells exhibited no reversion to the normal phenotype when injected with the anti-*ras* antibody. The finding that the v-*raf* oncogene, but not the receptor or receptor-coupled oncogenes, could bypass the inhibitory role of anti-ras antibodies suggested that Raf-1 either acted downstream of c-*ras* or through an independent signaling pathway. It should also be noted that stimulation of DNA synthesis and cell proliferation are achieved by microinjection of the Raf-1 oncogene protein into cells, but not by the nonactivated Raf-1 protein (Smith et al. 1990). The conclusive experiment implicating Raf-1 in the mitogenic signaling pathway was the suppression of cell transformation by inhibition of Raf-1 activity. Indeed, cells transformed by v-*ras* can be reverted to the normal phenotype either by Raf-1 antisense RNA or by a kinase-defective Raf-1 mutant (Kolch et al. 1991). The mutant, which has a single amino acid change in the ATP binding domain, can also revert ras transformation and block phorbol ester stimulation of cell growth. More striking, overexpression of Raf-1 regulatory domain can generate identical results (Kolch et al. 1991), arguing that PKC and c-*ras* lie upstream of c-*raf* in the mitogenic signaling pathway.

A number of signaling pathways have been implicated in Raf-1 activation, depending on the mitogen receptor system. In T cells, the antigen receptor (TCR) is devoid of tyrosine kinase activity but mediates its effects through the activation of two kinases of different specifities: PKC as a serine/threonine kinase and c-*fyn* as a tyrosine kinase (Samelson et al. 1986, 1990). In this system, stimulation of Raf-1 is entirely dependent on the PKC pathway (Siegel et al. 1990), and the enzyme is phosphorylated in serine only. In contrast, activation of Raf-1 by phosphorylation of tyrosine residues is obtained in T cells following stimulation of the interleukin 2 (IL-2) receptor by IL-2 (Turner et al. 1991). Interestingly, this receptor has recently been shown to be coupled to the tyrosine kinase c-*lck* which is a member of the same family of tyrosine kinases as c-*fyn* (Hatakeyama et al. 1991). Tyrosine phosphorylation of Raf-1 is also observed in myeloid cells after ligand activation of the IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) tyrosine kinase receptors (Carroll et al. 1990).

In murine fibroblasts as well as some other cell types such as macrophages, the role of tyrosine phosphorylation in Raf-1 activation is not clear

(see Li et al. 1991). Several mitogens have been tested in these systems including insulin, EGF, PDGF, and PKC after direct induction by 12-*O*-tetradecanoyl phorbol 13 acetate (TPA) (Morrison et al. 1988; Kovacina et al. 1990; App et al. 1991; Izumi et al. 1991; Baccarini et al. 1991). These studies show that activation through tyrosine kinase receptors is accomplished by serine phosphorylation of Raf-1 independent of the PKC activation by TPA (App et al. 1991; Baccarini et al. 1991). Earlier reports of the direct phosphorylation of Raf-1 by the PDGF receptor (Morrison et al. 1988, 1989) have been contradicted by later studies (Baccarini et al. 1990; Izumi et al. 1991) which suggest that Raf-1 activation following tyrosine kinase receptor stimulation is achieved by serine phosphorylation via an intermediate molecule. In agreement with this, Baccarini et al. (1990) showed that activation of Raf-1 in macrophages by colony-stimulating factor-1 (CSF-1) was through serine phosphorylation. More importantly, they demonstrated that Raf-1 was neither associated with the CSF-1 receptor in macrophages nor the PDGF receptor in 3T3 cells. They found that when cells are stimulated to proliferate at 4 °C a number of substrates become phosphorylated on tyrosine, with no increase of Raf-1 phosphorylation or activation. Evidence for a Raf-1 kinase molecule activated by insulin and other growth factors has recently been presented (Lee et al. 1991). Incubation of stimulated cell extracts with Raf-1 kinase expressed in *E. coli* led to the activation of Raf-1 autophosphorylation. Comparison of tryptic maps from in vitro and in vivo activated Raf-1 kinase show overlapping phosphorylated peptides suggestive of one or more kinase intermediates.

4.3 Cellular Targets of Raf-1 Kinase

The role of Raf-1 in cell growth appears essential; however, with the exception of Raf-1 itself no targets have as yet been identified for the kinase. Indeed, the substrates used today, including syntide, a putative autophosphorylation peptide, as well as histone, offer little insight into potential candidates as substrates. No consensus sequence has as yet been established for a Raf-1 phosphorylation site. However, substrates have been searched for on the basis of reports that transformation by v-*raf* alters gene expression patterns of cells (Heidecker et al. 1989). Substrate candidates of Raf-1 kinase implicated in these cellular modifications are transcription factor proteins. In transient transfection assays the Raf-1 oncogene has been shown to activate transcription from promoters containing the serum response element (SRE), the TPA response element, or the AP1/PEA1 transcription motif (Kaibuchi et al. 1989; Wasylyk et al. 1989; Jamal and

Ziff 1990). The transactivating effects are not observed with the protooncogene Raf-1 or with kinase-deficient v-*raf* obtained by point mutation of residue K_{53} involved in ATP binding. The promoters of the c-*fos* protooncogene and the β-actin gene contain elements responsive to Raf-1 transactivation (Kaibuchi et al. 1989; Jamal and Ziff 1990). These genes are known to be transcriptionally activated in the early response to mitogenic stimulation, a condition corresponding to Raf-1 kinase activation. Jamal and Ziff (1990) also suggest that the oncogenic form of Raf-1 may transform by constitutively activating early responsive protooncogenes such as c-*fos*.

5 MAP2 Kinase

MAP2 kinase, which catalyzes the phosphorylation of MAP2 in vitro, was first described as a serine/threonine kinase stimulated by insulin in serum-starved 3T3-L1 adipocytes (Sturgill and Ray 1986). Sturgill and coworkers were searching for an insulin-stimulated kinase in 3T3-L1 adipocytes which could phosphorylate inhibitor 2. In the phosphorylated form, this protein can no longer inhibit the activity of phosphatase 1. In their partially purified preparations of inhibitor 2, they fortuitously discovered a high molecular weight doublet, later identified as MAP2, which became phosphorylated by extracts from insulin-stimulated 3T3-L1 adipocytes. In subsequent studies, they used MAP2 as a substrate to follow the activity of the kinase during purification (Ray and Sturgill 1987, 1988). This kinase has been renamed mitogen-activated kinase (MAP kinase; Rossomando et al. 1989), myelin-basic protein kinase (MBP kinase; Sanghera et al. 1990), extracellular signal-regulated kinase (ERK; Boulton et al. 1990), and more recently as ribosomal protein S6 kinase kinase (RSK protein kinase; Chung et al. 1991). Unfortunately this random renaming is now causing some confusion to those not directly involved in the field. Following the earlier studies of Sturgill and coworkers, MAP2 kinase activity was also observed after quiescent human embryonic lung fibroblasts were induced to proliferate either with fresh fetal calf serum, EGF, PDGF, insulin-like growth factor-1, insulin, fibroblast growth factor, or phorbol esters directly activating PKC (Hoshi et al. 1988). Partial purification of MAP2 kinase induced by insulin in 3T3-L1 adipocytes indicated a protein of M_r 40000 phosphorylated on both tyrosine and threonine in vivo (Ray and Sturgill 1988).

5.1 Identification of MAP2 Kinase as pp42A

Two proteins with a similar molecular weight were identified in parallel studies aimed at the characterization of cytosolic proteins phosphorylated on tyrosine after mitogenic treatment. These proteins had M_r 42000 and 45000 on one-dimensional (Nakamura et al. 1983) or two-dimensional (Cooper and Hunter 1985) polyacrylamide gels, and each appeared to be made up of two distinct isoforms, phosphoproteins 42A and B (pp42A and B), and phosphoproteins 45A and B (pp45A and B). The acidic or A species may represent a more phosphorylated derivative of the B species. pp42A and MAP2 kinase comigrate on two-dimensional polyacrylamide gels, copurify on the same chromatographic column, and have corresponding patterns of proteolytic peptides (Rossomando et al. 1989); pp45 probably represents a related enzyme of the same kinase family (for a review see Sturgill and Wu 1991).

MAP2 kinases also appear to be activated during mitosis and meiosis. Tyrosine-phosphorylated proteins of M_r 42000 and 45000 were first detected in *Xenopus laevis* eggs arrested at M phase (Lohka et al. 1987; Cooper 1989). More recently it has been shown that MAP2 kinase is phosphorylated and activated during the M phases of meiotic and mitotic cell cycles in *X. laevis* (Gotoh et al. 1991). An activated MAP2 kinase, or in this case an MBP kinase, has also been purified from sea star oocytes (Sanghera et al. 1990). Antisera against the MBP kinase recognize two M_r 42000 phosphoproteins from *X. laevis* oocytes (Posada et al. 1991). Thus, it appears that members of the MAP2 kinase family are activated during mitosis and meiosis as well as during the transition from G_0 to G_1.

5.2 Activation of MAP2 Kinase

As stated above, activated MAP2 kinase is phosphorylated in both tyrosine residues as well as threonine/serine residues in vivo (Ray and Sturgill 1988; Cooper 1989). Treatment of MAP2 kinase with either phosphatase 2A, a protein phosphatase specific for phosphoserine and phosphothreonine residues (for a review see Ballou and Fischer 1986), or CD45, a phosphotyrosine-specific protein phosphatase, leads to complete inactivation of MAP2 kinase (Anderson et al. 1990). This has been confirmed by phospho-amino acid analysis of the protein which shows the specificity of the two phosphatases. The sites have been recently identified as a threonine and a tyrosine residue within the same tryptic peptide, separated by a single glutamic acid (Payne et al. 1991) and localized just upstream of the highly conserved A-P-E motif found in subdomain VIII of protein

kinases (Hanks et al. 1988). A number of cDNA clones for MAP2 kinase have been isolated from a rat brain cDNA library (Boulton et al. 1990, 1991). Two related clones, ERK1 and ERK2, encode proteins of M_r 43000 and 41200 (Boulton et al. 1991). Though both ERK1 and ERK2 contain the regulatory T-E-Y motif, only ERK2 contains a sequence identical to the phosphorylated peptide found in the MAP2 kinase, suggesting that ERK2 encodes the MAP2 kinase or pp42A. It should be noted that a MAP2 kinase of M_r 54000 (pp54 MAP2 kinase) has been recently isolated from the liver of cycloheximide-injected rats (Kyriakis and Avruch 1990). Although the sequence of this enzyme is not yet known, it is likely that this kinase is another member of the MAP2 kinase family, as it requires both tyrosine and serine/threonine phosphorylation for activity (Kyriakis et al. 1991). Interestingly, in contrast to MAP2 kinase, activation of the cdc2 serine/threonine kinase requires the dephosphorylation of two adjacent residues, a tyrosine and a threonine, located in the ATP-binding site of the catalytic domain, and the phosphorylation of a threonine residue just upstream of the A-P-E motif, in a site very similar to that of the threonine phosphorylated in MAP2 kinase (Lewin 1990).

Activation of MAP2 kinase is mediated by several mitogenic signaling pathways, e.g. the tyrosine kinase- and the PKC-mediated pathway mentioned earlier. Early reports on tyrosine phosphorylation of pp42 already indicated a role for PKC in the mechanism of activation (Kazlauskas and Cooper 1988; Vila and Weber 1988; Rossomando et al. 1989), though downregulation of PKC by prolonged phorbol ester treatment led only to attenuation of the MAP2 kinase response to growth factors, arguing that MAP2 kinase is activated by both PKC-dependent and PKC-independent pathways. The mechanism by which these different pathways lead to phosphorylation of MAP2 kinase on both threonine and tyrosine has been partially clarified. Initially, Ahn and coworkers mixed fractions from unstimulated and EGF-stimulated Swiss mouse 3T3 cells to identify factors involved in the activation of MAP2 kinases (Ahn et al. 1990; Ahn and Krebs 1990; Ahn et al. 1991). Using this approach, a factor referred to as MAP kinase activator, has been isolated. This activator stimulates MAP2 kinase activity in the presence of Mg^{2+} and ATP with concomitant phosphorylation of tyrosine and threonine residues in MAP2 kinase. However, there is no evidence that the MAP kinase activator is itself a kinase catalyzing MAP2 kinase phosphorylation (Ahn et al. 1991). Seger et al. (1991) have recently purified ERK1 from insulin-stimulated Rat1 cells and recombinant ERK2 from *E. coli* and showed that these MAP2 kinases autophosphorylate on both tyrosine and threonine residues. The autophosphorylation of ERK2 correlates with auto-activation of the kinase, although this activation occurs at a slower rate than in the presence

of MAP kinase activator. The data argue that autophosphorylation is part of the process of MAP2 kinase activation and that MAP2 kinase is phosphorylated on tyrosine as well as serine/threonine residues. The existence of kinases with tyrosine and serine/threonine specificity has recently been demonstrated (Ben David et al. 1991; Stern et al. 1991; Howell et al. 1991; Featherstone and Russell 1991). In the latter case, the product of the *wee1* gene, from *Schizosaccharomyces pombe*, which shows serine/threonine kinase structural features, is apparently able to autophosphorylate on tyrosine as well as serine residues and has been implicated as the tyrosine kinase which phosphorylates the cdc2 kinase (Featherstone and Russell 1991; Parker et al. 1991).

5.3 Cellular Targets of MAP2 Kinase

The target site for MAP2 kinase phosphorylation in myelin-basic protein has been identified as the threonine in the sequence P-R-T-P-P-P (Sanghera et al. 1990; Erikson 1990). This sequence is similar to those recognized by the cdc2 kinase (X-K-S/T-P-X; Langan et al. 1989) and a proline-dependent potein kinase (X-S/T-P-X; Vulliet et al. 1989). While no target has yet been identified for MAP2 kinase in vivo, the finding that it can phosphorylate and partially reactivate S6 kinase II in vitro has elecited great interest (Sturgill et al. 1988). S6 kinase II is a serine/threonine kinase implicated in the phosphorylation of ribosomal protein S6 during both meiotic maturation and the mitogenic response (see below). This kinase can be inactivated by incubation in vitro with either phosphatase 1 or phosphatase 2A (Sturgill et al. 1988). Partial reactivation and phosphorylation of S6 kinase II occurs in vitro with an insulin-stimulated MAP2 kinase, EGF-activated MAP2 kinases and MAP2 kinase activated during *X. laevis* oocyte meiotic maturation (Sturgill et al. 1988; Ahn et al. 1991; Haccard et al. 1990). Favoring a role for MAP2 kinase in phosphorylating S6 kinase II in vivo is the fact that MAP2 kinase reaches a peak of activation prior to S6 kinase II (Ahn et al. 1990; Ahn and Krebs 1990). However, while Sturgill et al. (1988) reported that activation of *X. laevis* S6 kinase II by MAP2 kinase was accompanied by threonine phosphorylation and the appearance of two new tryptic peptides, the tryptic phosphopeptide map of S6 kinase II labeled in vivo is more complex (Erikson and Maller 1989); this suggests that complete activation may require additional kinases. Three sequences similar to the site phosphorylated in MBP by MAP2 kinase are detectable in S6 kinase II, namely R-K-I-T-P-P, S-R-T-P-K-D, and S-K-P-T-P-L (Jones et al. 1988).

A second target of MAP2 kinase in vivo may be MAP2 itself. MAP2 is known to be a substrate for serine/threonine protein kinases in vitro, in-

cluding PKA, Ca^{2+}/calmodulin-dependent protein kinase, casein kinase I, and PKC, and for tyrosine-specific protein kinases such as the EGF receptor and insulin receptor. Phosphorylation of MAP2 catalyzed by these kinases results in a reduction in the ability of MAP2 to promote tubulin polymerization and to cross-link actin filaments. Indeed, Gotoh et al. (1991) recently reported that MAP2 kinase is activated during *X. laevis* meiotic maturation and could induce the interphase-metaphase transition of microtubule organization when added to *X. laevis* interphase extracts. Incubation of interphase extracts with the purified M phase-activated MAP2 kinase resulted in almost 50% reduction in the growth rate of microtubules and a steady-state length equivalent to that observed during metaphase. In considering potential cellular targets, it is interesting that the site in lamin which is phosphorylated by the cdc2 kinase in vitro leading to the breakdown of the nuclear envelope (Peter et al. 1990) is also a likely substrate for MAP2 kinase.

6 S6 Kinases

40S ribosomal protein S6 rapidly becomes phosphorylated on five serine residues in several biological systems in which quiescent cells are stimulated to proliferate, e.g. during hypertrophy after denervation of the rat diaphragm (Nielsen et al. 1982), regeneration of rat liver following partial hepatectomy (Gressner and Wool 1974), early fertilization of sea urchin eggs (Ballinger and Hunt 1981), meiotic maturation of *X. laevis* oocytes (Nielsen et al. 1982), and the stimulation of quiescent cells in culture by growth factors and oncogenes (Thomas et al. 1982). More important, this phosphorylation event is closely associated with in vivo- and in vitro-stimulation of protein synthesis (see below). These findings have made S6 an attractive model in the search for the kinase or kinases which modulate such responses. A number of kinases were previously implicated in the control of S6 phosphorylation, including PKA, PKC, Ca^{2+}/calmodulin-dependent protein kinase II, casein kinase I and a protease-activated kinase referred to as PAK (for a review see Kozma et al. 1989). However, most of these enzymes phosphorylate S6 to only a limited extent and their modes of activation are not consistent in all cases with a mitogen-induced signaling event.

A few years ago, a search for a mitogen-induced kinase eventually led to the discovery that extracts from serum-stimulated Swiss mouse 3T3 cells were up to 25-fold more potent in phosphorylating S6 than were equivalent extracts from quiescent cells (Novak-Hofer and Thomas 1984). More fun-

damental was the observation that full kinase activity during purification of the enzyme required the presence of ethyleneglycol-bis-(aminoethyl-ethene)N,N,N′,N′-tetraacetic acid (EGTA) and phosphatase inhibitors such as phosphotyrosine, phosphoserine, *p*-nitrophenyl phosphate, or *β*-glycerol phosphate in the extraction buffers (Novak-Hofer and Thomas 1985). The use of these compounds led to the identification of a kinase referred to here as the 70K S6 kinase (Jenö et al. 1988), which is activated by serine/threonine phosphorylation and selectively inactivated by a phosphatase of the 2A family (Ballou et al. 1988b). This enzyme is very specific for 40S ribosomal protein S6, with a K_m of 0.1 – 0.3 μM (H. Flotow and G. Thomas, in press), and, furthermore, it will phosphorylate four, possibly five, of the serine residues observed in vivo (Ferrari et al. 1991).

Employing a similar approach, Maller and colleagues detected two S6 kinases in extracts from *X. laevis* eggs arrested in the second metaphase of meiosis (Erikson and Maller 1985, 1986). These kinases of M_r 90000 and 92000 were termed S6 kinase I and II (S6K I and S6K II) according to their order of elution from a DEAE column. Both S6K II (Erikson and Maller 1986) and S6K I (Erikson and Maller 1991) have been purified, and shown to phosphorylate S6 in vitro, with an apparent K_m of 10 μM (S6K I) and 4 μM (S6K II). Thus, these kinases appear to be 15- to 30fold less effective than the 70K S6 kinase toward S6 as a substrate. Furthermore, they phosphorylate a number of substrates other than S6 (see below). Together with the group of R. Erikson, Maller and coworkers have generated antibodies to the cloned and expressed S6K II which recognize both S6K I and II, suggesting that they are related kinases (for a review see Erikson 1991). These antibodies made it possible to show unambiguously that the 90K family of S6 kinases is activated not only during meiotic maturation but also during G_0-G_1 transition of both chicken and mouse cells (Erikson 1991).

6.1 70K S6 Kinase

The relatively low abundance of the 70K S6 kinase compared to other kinases made it difficult to obtain enough protein from cell culture for sequencing (Jenö et al. 1989), but sufficient amounts of the kinase were subsequently obtained from the livers of rats treated with cycloheximide (Kozma et al. 1989a; Price et al. 1989). Cycloheximide is known to turn on many of the early mitogenic responses including transcription of c-*fos* and c-*myc* genes as well as S6 phosphorylation (Krieg et al. 1988). The effect of cycloheximide may be exerted at a level distinct from that of a general inhibition of protein synthesis (Mahadevan and Edwards 1991). Sequenc-

ing of tryptic peptides allowed the synthesis of oligonucleotides and eventually led to the screening of cDNA libraries. Two clones were obtained, one of 2.8 kb encoding a protein of M_r 56000 (Kozma et al. 1990) and a second of 2.3 kb encoding a protein almost identical to the first but which had a 23-aminoacid extension at the amino-terminus of the protein (Banerjee et al. 1990), resulting in a protein of M_r 56000. A clone similar to the latter one was also obtained by screening a rabbit cDNA library at low stringency with a probe against phosphorylase kinase (Harmann and Kilimann 1990). Expression of both clones in vitro and analysis of the products by polyacrylamide gel electrophoresis demonstrated that the 2.8-kb clone yields a protein identical in size to the purified 70K S6 kinase, whereas the 2.3-kb clone with a larger open reading frame yields two products of 85K and 70K (C. Reinhard et al., in press). The two cDNA clones differ at the first nucleotide upstream of the AUG start site of the 70K clone; however, their sequence is identical throughout the 70K-coding region and the 3′ untranslated region, arguing that the two transcripts originate from the same gene. Four transcripts can be detected by northern blot analysis using a probe encompassing the coding region of the 70K S6 kinase. By producing specific probes against unique sequences at the 5′ end of each clone, it was possible to show which transcript corresponds to which clone, and that both clones are derived from the same gene (C. Reinhard et al., in press).

The 70K S6 kinase can be divided into three distinct regions: (1) A short amino-terminal region of 40–45 amino acids which is extremely acidic and perhaps involved in substrate recognition (H. Flotow and G. Thomas, in press); (2) a central catalytic domain containing all the hallmarks of a serine/threonine kinase (Kozma et al. 1990) and having approximately 45% homology with PKC, placing the 70K kinase in the second messenger family of protein kinases (Hanks et al. 1988); and (3) the carboxy-terminal half of the molecule containing the putative regulatory domain based on the fact that all the phosphorylation sites are located at this end of the molecule (S. Ferrari, N. Totty and G. Thomas, unpublished data). It has been suggested that this domain may also contain a pseudosubstrate sequence for the kinase (Banerjee et al. 1990), similar to those described above for PKA and PKC. There is, however, little homology between the putative pseudosubstrate sequence and the carboxyl end of S6. Indeed the K_i of this peptide is 60-fold lower than that of a peptide derived from the phosphorylation sites of S6 (H. Flotow and G. Thomas, in press).

6.2 90K S6 Kinase Family: S6 K I and II

To obtain protein sequence data for the cloning of S6 K II, 100 μg of the enzyme were purified from 1.2 kg of unfertilized *X. laevis* eggs (Jones et al. 1988). Ten tryptic peptides were isolated and sequenced, allowing the generation of oligonucleotide probes for screening a *X. laevis* ovarian cDNA library. Two cDNA clones were obtained which encoded kinases of M_r 83000 and 70000, referred to as S6 K IIα and S6 K IIβ. The proteins were highly homologous in the coding region, having 95% identity (Jones et al. 1988). Surprisingly, the amino acid sequence revealed two catalytic domains, with the amino-terminal domain closely related to PKA and the carboxyl-terminal domain similar to phosphorylase kinase. It remains unclear which of the two kinase domains is involved in the phosphorylation of S6, or whether both are involved (Erikson 1991). However, it should be noted that phosphorylase kinase does not phosphorylate S6 in vitro, nor does phosphorylase β serve as a substrate for S6 K II. In contrast, PKA phosphorylates S6 and S6 K II phosphorylates glycogen synthase and troponin, two substrates phosphorylated by PKA (Erikson and Maller 1988). Finally, the 70K S6 kinase is approximately 56% homologous with the PKA-like domain of S6 K IIα. These findings suggest that it is the PKA-like kinase domain which is responsible for carrying out S6 phosphorylation.

Three possibilities could explain the existence of two highly related gene products, S6 K IIα and S6 K IIβ in *X. laevis*. The two kinases may have resulted from divergence following the genome duplication of *X. laevis* 30 million years ago, it is also possible that the *X. laevis* population itself is heterozygous at a single locus which encodes the two gene products or that the S6 K II gene products represent a gene family, as described above for PKA, PKC, MAP2 kinase and Raf-1 kinase (Jones et al. 1988). The recent report of multiple gene families in other species supports the latter possibility (Alcorta et al. 1989). Several closely related genes were isolated from mouse and chicken libraries using *X. laevis* S6 K IIα as a probe (Alcorta et al. 1989). The gene products were named rsk for ribosomal S6 kinase. The fact that S6 K II is related to S6 K I also supports the notion that the two 90K kinases probably represent a large gene family unlike 70K S6 kinase (Erikson et al. 1991).

6.3 S6 Kinase Activation

The 70K and 90K S6 kinases are both activated by serine/threonine phosphorylation and removal of these groups returns the proteins to the inac-

tive state (Ballou et al. 1988b, c; Sturgill et al. 1988). As mentioned earlier, in the case of S6 K II the activation is mediated, at least in part, by MAP2 kinase. However, two lines of evidence suggest that the 70K S6 kinase lies on a distinct signaling pathway. First, as noted earlier, cycloheximide activates the S6 kinase in the whole animal and in cultured cells (Gressner and Wool 1974; Blenis and Erikson 1986). Antibodies generated against bacterially expressed S6K II, however, were not able to immunoprecipitate S6K II kinase activity from extracts of cycloheximide-treated cells, suggesting that only the 70K S6 kinase is activated by cycloheximide (Erikson 1991). Second, MAP2 kinase is not able to reactivate or phosphorylate the 70K S6 kinase following partial or complete inactivation by phosphatase 2A (Ballou et al. 1991). More important, insulin appears to have little or not effect on MAP2 kinase activation in Swiss 3T3 cells, whereas EGF raises basal levels 50- to 100-fold (Ballou et al. 1991). In contrast, both mitogens activate the 70K S6 kinase to approximately the same extent. Obviously the identification of the 70K S6 kinase kinase will be facilitated by the identification of the sites of phosphorylation leading to its activation.

The stimulation of S6 kinase activity in Swiss 3T3 cells treated with EGF is biphasic (Šuša et al. 1989). Chronic treatment of cells with TPA leads to downregulation of PKC and almost a complete loss of the late phase of S6 kinase activation with no effect on the early phase. Almost identical results were obtained with a specific inhibitor of PKC, CGP41 251 (M. Šuša et al., in press). Furthermore, the kinase has been purified from both phases and found to represent the same 70K S6 kinase (Šuša and Thomas 1990). These results would suggest that the late phase of S6 phosphorylation is under the control of PKC. However, insulin and PDGF also induce biphasic S6 kinase activation, but in neither case is the late phase response altered by pretreatment with TPA or CGP 41 251 (M. Šuša et al., in press). These results argue that, depending on the mitogen, the late phase response is controlled by both PKC-dependent and PKC-independent signaling pathways.

A time-dependent, biphasic activation of S6 phosphorylation has also been observed in secondary chicken embryo fibroblasts (Sweet et al. 1990a, b). Furthermore, antibodies to S6K II and partial purification studies have shown that S6 K II is a major contributor to the early phase of activation but much less to the late phase (Sweet et al. 1990b). Similar results have recently been obtained in 3T3 cells (Chen and Blenis 1990; Chen et al. 1991; Chung et al. 1991). In the latter studies it was argued that S6K II activity could only be detected after immunoprecipitation and not in whole extracts (Chen and Blenis 1990). Obviously, it will be of interest in the future to determine the role each S6 kinase plays in the phosphorylation of S6.

6.4 Targets of 70K S6 Kinase and S6 KII

As stated above, the strategy in purifying these two kinases was to search for an enzyme which multiply phosphorylates S6 in vitro. Sufficient amounts of highly phosphorylated S6 were purified recently from the livers of cycloheximide-injected rats to allow the identification of these sites. Five sites of phosphorylation are clustered in a 15-amino-acid segment at the carboxy end of S6 (Krieg et al. 1988). The sites phosphorylated included S_{235}, S_{236}, S_{240}, S_{244} and S_{247} and appear to be phosphorylated in a specific order: $S_{236} > S_{235} > S_{240} > S_{244} > S_{247}$ (Krieg et al. 1988). We have recently obtained identical results for S6 derived from serum-stimulated cells (H.R. Bandi and G. Thomas, unpublished data). In vitro the 70K S6 kinase will phosphorylate the first four sites and possibly the fifth (S. Ferrari et al. 1991). Similar results have been obtained for S6K II (R.E.H. Wettenhall and J.L. Maller, personal communication). From in vitro and in vivo data it would appear that the late sites are involved in the activation of protein synthesis and that they alter the pattern of translation (Palen and Traugh 1987). In contrast, the early sites may regulate late-site phosphorylation. This finding is consistent with the localization of S6 in the mRNA-tRNA binding site of the 40S ribosome and the fact that antibodies derived against a peptide containing the sites of S6 phosphorylation block mRNA binding (R.E.H. Wettenhall, personal communication).

In an effort to define the substrate recognition determinants for the 70K S6 kinase, we have generated a number of synthetic peptides. The K_m for a 20-amino-acid peptide from K_{230} to K_{249} is almost as good as for 40S ribosomes less than 1 μM (H. Flotow and G. Thomas, in press). By constructing specific derivatives of this sequence we were able to show that the critical sites for recognition reside in the block of basic amino acids K-R-R-R, with the second and fourth arginine being absolutely required for kinase recognition. The spacing of the target serine is also critical, two amino acids being required between the last arginine and the serine. A spacing of one or three amino acids eliminated the peptide as a substrate for the kinase.

In contrast to 70K S6 kinase, S6K II has a much wider substrate specificity, readily using, for instance, the peptide L-R-R-A-S-L-G, a typical PKA substrate which the 70K S6 kinase will not phosphorylate. The S6K II will also phosphorylate glycogen synthase, troponin I, the G subunit of phosphatase 1, and lamin C. The last substrate is particularly interesting in that this protein becomes heavily phosphorylated during meiotic maturation in parallel with a 10- to 20-fold activation of S6K II. Such phosphorylation leads to breakdown of the nuclear envelope and arrest in the second meta-

phase of meiosis. It will be of interest to determine whether the sites of phosphorylation catalyzed by S6 KII kinase play a role in this process.

7 Other Kinases and Future Perspectives

Several other serine/threonine kinases have been implicated in the activation of cell growth, including casein kinase II (Sommercorn and Krebs 1987; Klarlund and Czech 1988; Ackerman and Osheroff 1989), insulin-stimulated kemptide kinase (KIK) (Klarlund et al. 1990, 1991), Ca^{2+}/calmodulin-dependent kinase III (Palfrey et al. 1987), and possibly the proto-oncogene c-*mos* (Sagata et al. 1988, 1989). Although these kinases have not attracted as much attention as those discussed above, they may also play key roles in the activation of cell growth. Indeed, the number of newly discovered serine/threonine kinases has been growing at an exponential rate and many key enzymes may still await identification. What is clearly lacking in many cases of known kinases is identification of the substrate. The existence of unique substrates will make the search much more difficult. Beyond the identification of the substrates, the next challenge is defining the exact role of these kinases in the mitogenic response, as well as describing how the kinases crosstalk with one another. The first part of this question has been elegantly approached in the case of the Raf-1 kinase by the use of dominant-negative mutants. In the future, this approach, together with "gene-knockout" studies by homologous recombination, should greatly advance our knowledge of the mechanisms involved in propagating the mitogenic response through serine/threonine kinases.

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Rev. Physiol. Biochem. Pharmacol., Vol. 119
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Structure Function of the Growth Factor-Activatable Na^+/H^+ Exchanger (NHE1)

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1 Introduction

The Na^+/H^+ exchanger is a plasma membrane transporter which is involved in intracellular pH (pH_i) regulation. This transporter catalyzes an H^+ extrusion coupled to Na^+ influx. The inward directed Na^+ gradient that is produced by the Na^+/K^+-ATPase provides a constant driving

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force for H^+ extrusion. The Na^+/H^+ antiport was first demonstrated to function in vesicles from the brush borders of rabbit kidney and small intestine by Mürer et al. (1976). Since that time, the existence of this transporter has been described in many animal cells including fibroblasts, smooth, skeletal, and cardiac muscle, and blood, epithelial, and neuronal cells. It is now well known that the Na^+/H^+ exchanger is ubiquitously distributed in virtually every cell from prokaryotic to eukaryotic cells (Grinstein 1988; Grinstein et al. 1989; Seifter and Aronson 1986 for review). In addition to the Na^+/H^+ exchanger, pH_i is also regulated by several other plasma membrane H^+ extrusion systems, including Na^+-dependent and -independent Cl^-/HCO_3-exchangers and an ATP-dependent H^+ pump (Madshus 1988 for review). The relative contribution of these H^+ extrusion systems to the regulation of pH_i greatly depends on the cell type under study.

In addition, the Na^+/H^+ exchanger serves as a major Na^+ entry pathway in many cell types. Moreover, net sodium influx could influence the water balance between the intra- and extracellular spaces. Therefore, Na^+-dependent transporters such as the Na^+/H^+ exchanger have a major role in the regulation of intracellular volume (Grinstein et al. 1989; Seifter and Aronson 1986 for review). Finally, in epithelial cells, the exchanger is involved in net transepithelial H^+ and Na^+ secretion. It has been repeatedly documented that the primary functions of the exchanger, namely, intracellular pH, Na^+ and volume regulation, are intimately related to physiological and pathophysiological cellular events including fertilization, cell cycle control, differentiation, essential hypertension, kidney diseases, and hypertrophy (Grinstein et al. 1989; Mahnensmith and Aronson 1985 for review). Among these putative roles the most extensively studied function of the exchanger is its contribution on the control of cell proliferation (Grinstein et al. 1989 for review). Intracellular pH critically controls the entry of quiescent cells into the cell cycle. For example, in the absence of bicarbonate, the exchanger-deficient mutant of Chinese hamster fibroblast fails to reinitiate DNA synthesis and to proliferate in an extracellular pH of less than 7.2 (Pouysségur et al. 1985). Simply, an elevation of pH_i by 0.2 units is sufficient to restore a full mitogenic response.

In accordance with the physiological implications described above, a prominent feature of the Na^+/H^+ exchanger is that it is rapidly activated in response to a variety of mitogenic and nonmitogenic signals such as growth factors, oncogenes, sperm, neurotransmitters, hormones, chemotactic peptides, phorbol esters, lectins, and osmotic change (Grinstein et al. 1989 for review). This activation can be detected by either an increase in Na^+ flux which is amiloride-sensitive, or by amiloride-sensitive or Na^+-dependent cytoplasmic alkalinization. For example, addition of

growth factors to quiescent fibroblasts results in a persistent cytoplasmic alkalinization (0.15 to 0.3 pH units) in the absence of bicarbonate. It is well accepted that this exchanger activation occurs through the increased affinity for the intracellular H^+ at an "H^+ modifier" site (Moolenaar et al. 1983; Paris and Pouysségur 1984) which is thought to be distinct from Na^+ and H^+ transport sites (Aronson et al. 1982). However, the molecular mechanism of this activation remains to be elucidated at the level of the exchanger molecule itself. It is also unknown how distinct types of signals result in the same final consequence, the activation of the Na^+/H^+ exchange. In this context, the exchanger represents an interesting model to study signal transduction.

In a broader context of ion transporters, the Na^+/H^+ exchanger is of interest since it requires neither the energy from ATP hydrolysis nor the formation of covalent phosphoester bonds as other cation ATPases do during the transport cycle, but instead, requires H^+ binding at an "H^+ modifier" site(s) for full exchange activity. It catalyzes the exchange of Na^+ and H^+ in either direction with a stoichiometry of one to one. Therefore, the exchanger clearly differs from the ATP-dependent cation pumps such as the Na^+/K^+-ATPase and the Ca^{2+}-ATPase in that they undergo large changes in chemical and vectral specificity toward their ligands. In this context, the Na^+/H^+ exchanger molecule, whose function solely depends on the concentration of Na^+ and H^+, represents one of the simplest models to understand the basic principles of the energy transduction mechanism of ion transport by which chemical or physical energy (ATP, light, ...) is transduced into chemiosmotical work.

Recently, the complementary DNA of the human Na^+/H^+ exchanger was cloned and its primary sequence deduced (Sardet et al. 1989). The success of molecular cloning and production of specific antibodies gave us a starting point to challenge many interesting questions concerning structure function of this molecule: (1) How do distinct signaling pathways activate the exchanger? (2) Is direct modification of the exchanger involved in this activation? (3) If so, which part of the molecule is modified? (4) What is the quaternary structure of the exchanger and the basic functional unit? (5) Which part of that molecule forms the amiloride binding site? (6) Which part of the molecule is necessary for intracellular H^+ sensing. (7) How does the binding of the H^+ ion at modifier sites induces the conformational change of the protein which turns on the exchanger? (8) Where are the Na^+ and H^+ translocation sites? (9) How many isoforms of the exchanger exist?

Using the developed experimental tools, studies to answer these questions are underway. In this report, we will present the most recent progress in determining the structure-function relationship of the Na^+/H^+ ex-

changer and its molecular mechanism of activation in response to extracellular signals.

2 Primary, Secondary, and Quaternary Structure

2.1 Molecular Cloning of Human Na^+/H^+ Exchanger

In the past decade, much effort was made to identify the molecular entity of the Na^+/H^+ exchanger. Efforts were hampered by a lack of both suitable specific antibodies and good ligands for affinity purification. Indeed, despite several attempts, labeling studies using chemical modifiers and photoreactive amiloride analogues along with in vitro reconstitution, unfortunately, did not lead to decisive molecular identification.

The strategy that finally allowed the identification of the Na^+/H^+ exchanger consisted of several steps including; (1) selection of a stable exchanger-deficient mouse fibroblast cell line, (2) transfection of this mutant cell line with genomic DNA obtained from exchanger-competent human cells and selection of the cells overexpressing the human exchanger, and (3) the molecular cloning of the human transfected gene coding for the human Na^+/H^+ exchanger.

The strategy for selection of the exchanger-deficient or overexpressing cells which is generally called "H^+ suicide" or "H^+ killing" methods, respectively, is based on the Na^+/H^+ exchange reversibility and the toxicity of a high concentration of cytosolic H^+. To select mutants lacking Na^+/H^+ antiport activity, fibroblast cells (Chinese hamster lung or mouse fibroblasts) were loaded with Li^+ and then exposed to a Na^+ and Li^+-free medium at an acidic extracellular pH (e.g., $pH_o = 5.5$). Under such conditions cell viability drops within a few minutes as a consequence of rapid H^+ uptake by the cells which possess the exchanger. Thus, exchanger-deficient cells resistant to this H^+-suicide test were isolated from a cell population chemically mutagenized (Pouysségur et al. 1984; Pouysségur 1985). On the other hand, for the selection of mutants with an altered or overexpressed level of the exchanger, we can use the ion gradients of Na^+ and H^+ in a manner opposite to that for selection of antiporter-deficient mutant cells. In this case, the cells are first loaded with H^+ by an NH_4^+ prepulse technique and cells are exposed to a Na^+-containing medium (Fig. 1). If H^+ efflux is slowed down during the second incubation by lowering the concentration of Na^+ or by adding an amiloride analogue (e.g., 5-*N*-methyl, *N*-propyl amiloride, MPA), the cells do not survive unless they overexpress the exchanger or have a mutated exchanger which

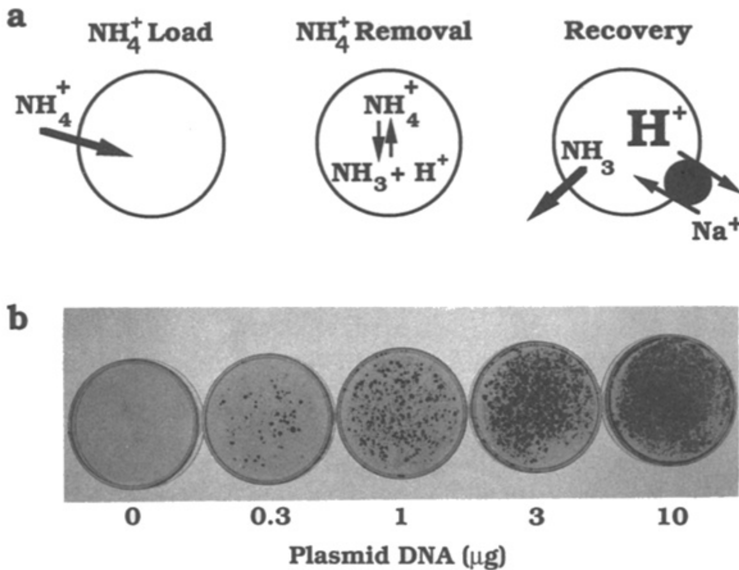

Fig. 1a, b. Schematic representation of H^+-killing selection of Na^+/H^+ exchanger-expressing cells. **a** Principle of H^+-killing selection. The cells are initially loaded with NH_4^+ for 1 h at 37 °C in a medium containing 50 m*M* NH_4^+Cl (*NH_4^+ load*). Loaded NH_4^+ is equilibrated with NH_3 and H^+ in the cytosol. If the extracellular NH_4^+ is removed by washing NH_4^+-free medium (*NH_4^+ removal*), the intracellular concentration of H^+ rapidly increases because NH_3 selectively and freely goes out of the cells. This acid load is lethal for the cells which do not have the H^+ extrusion system. If the cells possess the Na^+/H^+ exchanger, they can recover from this acid load (*Recovery*). Thus, we can select the cells that express the Na^+/H^+ exchanger. **b** Cells transfected with the exchanger cDNA and selected by H^+-killing method. The exchanger-dificient cell line PS120 cells (2×10^5 cells/60 mm per dish) were transfected with 0 to 10 ug of human Na^+/H^+ exchanger cDNA cloned into an eukaryotic expression vector pECE which contains SV40 early promoter. The H^+-killing test was repeated five times over a period of 3 weeks. The number of cell colonies stably expressing the exchanger proportionally increases with increasing concentration of plasmid DNA initially used

has a lower affinity for the amiloride analogue (Franchi et al. 1986a; Pouysségur et al. 1984).

By using these strategies for cell selection, a mouse cell line which expresses the human Na^+/H^+ antiporter was obtained (Franchi et al. 1986b). From this cell line, a 0.8-kb genomic probe was isolated and used for cDNA cloning of the Na^+/H^+ exchanger. The clone obtained from the mouse transformant consisted of 3977 bp and contained a 5′-noncoding sequence of 407 bp, a 2445-bp open reading frame and a 3′-noncoding sequence of 1125 bp.

2.2 Primary and Secondary Structure

The nucleotide sequence of the Na^+/H^+ exchanger cDNA predicts a protein of 815 amino acids (molecular weight = 90704). A hydropathy plot of the amino acid sequence by the method of Engelman et al. (1986) or of Kyte and Doolittle (1982) revealed that the exchanger contains 10 or 12 putative transmembrane spanning segments. Figure 2 shows a hypothetical model of the Na^+/H^+ exchanger obtained from the program of Kyte and Doolittle (1982). One striking structural feature is that the exchanger consists of two distinct domains: (1) The amphipathic NH_2 terminal domain with 500 amino acids, which consists of four major extracellular hydrophilic loops, two of them (the first and fourth loops) are very hydrophilic and contain putative N-linked glycosylation sites (Asn-75 and Asn-370); (2) the highly hydrophilic C-terminal domain, which likely represents a large cytoplasmic domain of the molecule. In fact, this domain has been suggested to be facing the cytoplasm by the fact that an antiserum which was raised against this portion cannot gain access to the epitope from the extracellular medium (Sardet et al. 1990). This C-terminal domain contains several potential phosphorylation sites.

The transmembrane configuration, which contains 10 to 12 transmembrane spanning helices, allows a comparison to be made to the growing family of transporter molecules: the Na^+-independent Cl^-/HCO_3-exchanger (band 3; Kopito and Lodish 1985); the Na^+/Ca^{2+} exchanger (Nicoll et al. 1990); the Na^+-dependent (Hediger et al. 1987) and -independent glucose transporters (Mueckler et al. 1985; Thorens et al. 1988); the *Escherichia coli* Na^+/H^+ exchanger (Karpel et al. 1988); and H^+ symporters for lactose, arabinose, and xylose (Buche et al. 1980; Maiden et al. 1987). It is interesting that the pronounced dichotomy between the large hydrophilic and amphipathic regions of the Na^+/H^+ exchanger is also shared by the Cl^-/HCO_3-exchanger (Kopito and Lodish 1985) and the Na^+/Ca^{2+} exchanger (Nicoll et al. 1990). However, the Na^+/H^+ exchanger of *E. coli* has a smaller hydrophilic domain (Karpel et al. 1988).

2.3 Isoforms of Na^+/H^+ Exchanger

The above first-described exchanger cDNA cloned from human belongs to the "housekeeping" type of the exchanger, which is now referred to as NHE1 (Na^+/H^+ exchanger 1). This form is apparently expressed in most cell types, present only in the basolateral membranes of polarized epithelial cells, sensitive to amiloride and its derivatives (5-amino substituted), and growth factor activatable. The cDNA coding for the Na^+/H^+ exchanger

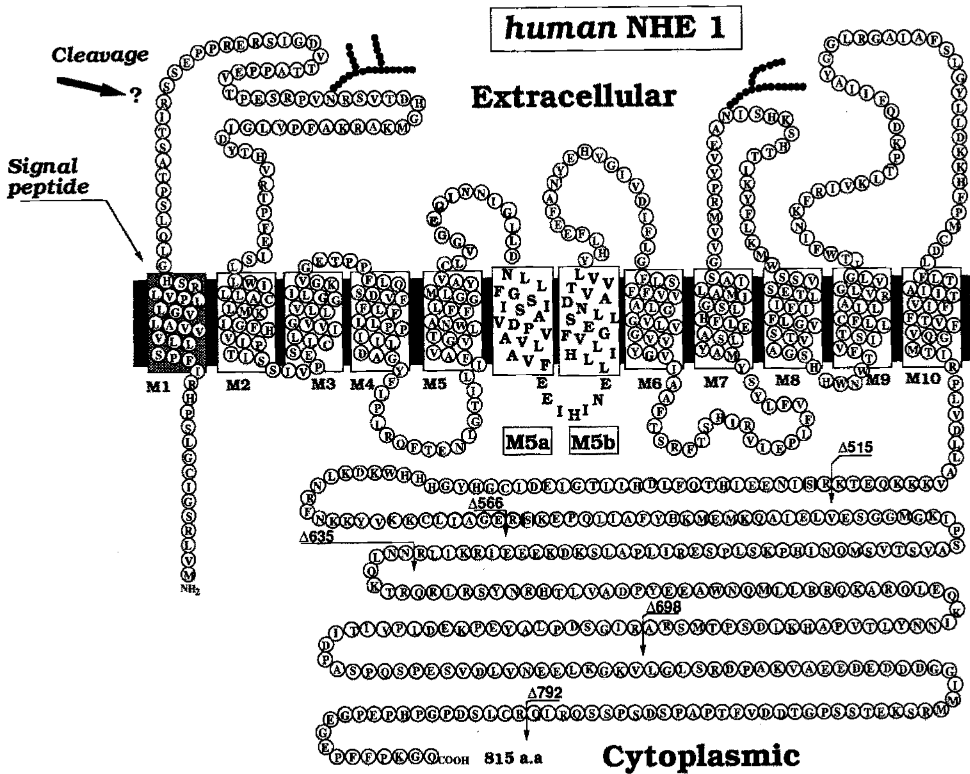

Fig. 2. Primary amino acid sequence and topological structure model of human Na^+/H^+ exchanger. Amino acid sequence was deduced from nucleotide sequence of human Na^+/H^+ exchanger cDNA and it is represented in the figure by the *single letter code.* Hydrophobicity plot of the amino acid sequence by method of Engelman et al. (1986) predicts that the exchanger consists of the NH_2-terminal amphipathic portion which contains ten putative transmembrane spanning segments (*M1* to *M10*), followed by the large hydrophilic C-terminal portion which is thought to be facing the cytoplasmic side. There are two putative N-linked glycosylation sites (Asn75 and Asn370) in the first and fourth extracellular loops. *Arrows* show the location within the C-terminal cytoplasmic domain at which the truncation of the molecule was carried out. For example, the deletion mutant $\Delta 515$ contains N-terminal 515 amino acids, but loses the region between amino acid 516 and 815. Note that the putative transmembrane segment M5a and M5b (12 segments model) are the most highly conserved region between 4 Na^+/H^+ antiporter isoforms so far cloned (Tse C-M, Brant S, Walker S, Pouysségur J, Donowitz M (1992) J Biol Chem (in press); Orlowksi J, Shull G, personal communication). Transmembrane segment M1 is not necessary for ion transport and might be cleaved as a signal peptide in NHE1 isoform (our unpublished results)

has been isolated from several different mammalian species, including rat (D. Pearse 1991, personal communication), rabbit (Tsé et al. 1991; Fleigel et al. 1991; Hildebrandt et al. 1991), pig (Reilly et al. 1991), and hamster (Counillon et al., in preparation). Their amino acid sequences displayed strong homology (>90%) with the human NHE1. In addition, the NHE1 of these animal species was detected by the specific antibody directed against human NHE1 (RP-c28). Recently, the human NHE1 gene has been characterized (promoter and regulatory sequences, intron/exon organization); it is at least 70 kb long with 12 exons (Miller et al. 1991) and is located on chromosome 1, locus p34–36 (Mattei et al. 1988).

Another spatially and pharmacologically distinct exchanger has been recently described in kidney (Haggarty et al. 1988) and intestinal epithelial cells (Knickelbein et al. 1988). This type of antiporter is produced during polarization of epithelial cells and finally inserted into the apical membrane (Viniegra and Rabito 1988). Recently, a functionally and structurally related isoform (NHE2) has been cloned by low stringency screening of a rabbit cDNA library with an NHE1 cDNA probe (Tsé et al., in preparation). The NHE2 isoform which consists of 780 amino acids is strongly expressed in small intestine and kidney cortex and less sensitive to 5-amino substituted amiloride analogues than NHE1. Like NHE1, NHE2 consists of an N-terminal amphiphilic domain which contains between 10–12 putative transmembrane spanning segments, followed by a large C-terminal hydrophilic domain which must face the cytoplasm. The amino acid sequence of rabbit NHE2 is 60% homologous to that of rabbit NHE1 in the N-terminal amphiphilic domain, while there is only 34% homology within the C-terminal hydrophilic domain. Future structure-function study will reveal which portion of the molecule is essential of the sorting out of the two isoforms of the exchanger either into the basolateral or apical membrane of epithelial cells.

More recently, a novel type of Na^+/H^+ exchanger which is pharmacologically different from both NHE1 and NHE2 has been described in cultured hippocampal neurons (Raley-Susman et al. 1991). Surprisingly, this type of exchanger is inhibited by neither amiloride nor its more potent 5-amino-substituted analogues and could therefore represent at third type of exchanger isoform. Recently, cDNA coding for an Na^+/H^+ exhanger has been cloned from trout (*Salmo gairdneri*) (Borgese et al., in preparation) and *Caenorhabditis elegans* and *Caenorhabditis briggsae* (Prasad et al. 1991). The partial nucleotide sequence of the *C. elegans* Na^+/H^+ exchanger cDNA revealed that 36% of the 300 amino acids identified from the partial nucleotide sequence are identical within amino acids 328 to 627 of the human Na^+/H^+ exchanger protein sequence. It is unknown which mammalian isoform corresponds to these genes isolated from nonmam-

malian species. At any rate, the amino acids which are conserved in the *C. elegans* or trout antiporter which is phylogenically distinct from mammalian species must be important for the function of the exchanger.

2.4 Glycosylation

The NHE1, like many other plasma membrane ion transporters, has been demonstrated to be a glycoprotein by the following observations: (a) The apparent molecular weight of the in vitro translated NHE1 cRNA product on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was close to that calculated from the amino acid sequence, probably because of the absence of posttranslational modification in the system used (Sardet, unpublished observation); (b) When NHE1 expressed in the hamster fibroblast was immunoprecipitated with the specific antiserum and analyzed by SDS-PAGE, the apparent molecular mass (110 kDa) was higher than the value calculated from the complete amino acid sequence. Treatment with neuraminidase and endoglycosidase F, but not endoglycosidase H reduced the apparent size of the protein from 110 to 90 kDa; (c) The functional antiporter has been expressed in the plasma membrane of insect cells (Sf9 cells) using a baculovirus expression system (Fafournoux et al. 1991; Fig. 3). The molecular mass of the exchanger expressed in this system was approximately 85 kDa, which accounts for the non-glycosylated form (Fig. 3). Thus, the exchanger is glycosylated through N-linkage, consistent with the existence and the conservation of two potential N-linked glycosylation sites in the amino acid sequence (Asn 75 and Asn 370; see Fig. 2). The experiment in Sf9 cells suggests that N-linked oligosaccharides are not essential for the exchange activity of NHE1 (Fig. 3).

The effect of removal of N-linked oligosaccharides on the exchanger function has been examined in rat renal brush-border membranes (Yusufi et al. 1988). Treatment of this membrane with endoglycosidase F, but not endoglycosidase H, partially reduced the maximal rate (V_{max}) of exchange without changing the apparent K_m value for Na^+. Hence, although the exchanger may function without N-linked oligosaccharides, this posttranslational modification may play a role in regulation of the exchange activity of NHE2.

2.5 Quaternary Structure

It is well accepted that many ion transporters exist in an oligomeric form within the membrane. For example, studies on the erythrocyte anion ex-

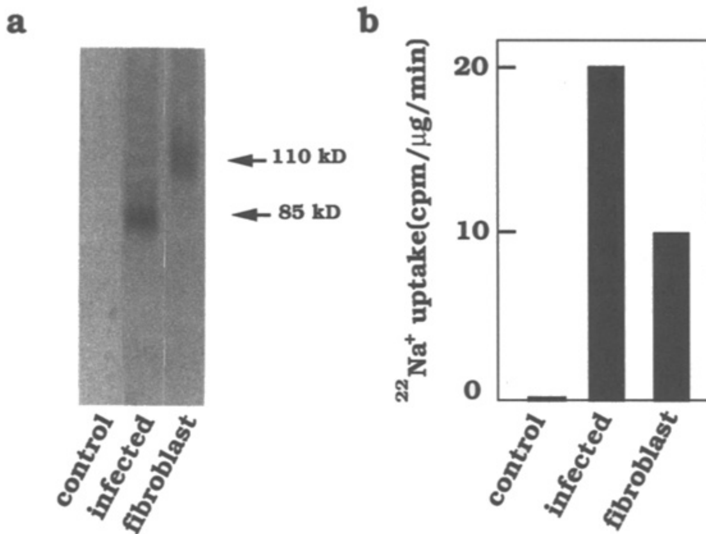

Fig. 3 a, b. Immunological (**a**) and functional (**b**) detection of the Na^+/H^+ exchanger expressed in insect and fibroblastic cells. **a** Sf9 cells were infected with recombinant baculovirus. Two days after transfection, the proteins were electrophoresed, blotted onto the nitrocellulose, filter and the antiporter detected with the specific antibody raised against the C-terminal hydrophilic domain. **b** In parallel with immunoblot, the functional Na^+/H^+ exchange activity expressed in the plasma membrane of Sf9 cells was evaluated by measuring amiloride-sensitive $^{22}Na^+$ uptake. By comparison, immunoblot and $^{22}Na^+$ uptake activity are also shown for hamster fibroblasts overexpressing the exchanger by 15-fold. The apparent molecular weight of the exchanger expressed in Sf9 cells is smaller than that in fibroblasts, due to the lack of glycosylation in Sf9 cells

changer provide substantial evidence for oligomeric structure, although there is no conclusive evidence for the functioning of the oligomer (Jennings 1989). Recently, Otsu et al. (1989) have made transient kinetic measurements of Na^+/H^+ exchange at 0 °C in renal brush border membrane vesicles. They observed that $^{22}Na^+$ uptake consisted of an initial burst phase preceded by a short induction phase and followed by a late, steady state phase. The Na^+ concentration dependence of the amplitude of the burst phase, but not the steady state rate, exhibited sigmoidal behavior, suggesting that the Na^+/H^+ exchanger (probably NHE2) works as an oligomer with at least two Na^+ transport sites which interact cooperatively, though one cannot exclude the possibility that two cooperative Na^+ binding sites per monomer exist. Otsu et al. (1989) concluded that a simple saturation kinetics during steady state was due to a flip-flop mechanism of oligomers.

The following data provide direct evidence to suggest that NHE1 exists as a dimer within the plasma membrane: (a) When the ^{32}P-labeled an-

tiporter was immunoprecipitated with specific antibodies (RP28), in addition to the major band of 110 kDa, faint phosphorylated band appeared in the slightly higher molecular mass range of 200 kDa. This band might account for the oligomeric form the exchanger. (b) Treatment of the cells with a cross-linker, disuccinimidylsuberate (DSS), shifted almost all of the 100 kDa band to the higher molecular weight form (Fafournoux et al., in preparation). These findings support the notion that NHE1 exists in the membrane as a dimer, but further experiments are required to answer to whether the functional unit is a monomer or an oligomer (e.g., dimer, trimer).

3 Functional Domain Structure

3.1 Two Major Functional Domains

A prominent structural feature of the Na^+/H^+ exchanger is that it can be separated into two large portions, the NH_2-terminal amphiphilic domain and the COOH-terminal hydrophilic cytoplasmic domain. To study the structure function of the antiporter, a set of cDNA deletion mutants within the cytoplasmic domain have been generated (Fig. 2) and expressed in the antiporter-deficient cell line PS120. Interestingly, complete removal of the cytoplasmic domain preserved amiloride-sensitive Na^+/H^+ exchange activity (Fig. 4), indicating that the NH_2-terminal transmembrane domain is both sufficient for insertion of the antiporter into the plasma membrane and for the exchange activity (Wakabayashi et al. 1991). Furthermore, deletion of the complete cytoplasmic domain (1) markedly shifted the pK value for cytosolic H^+ to the acidic range but preserved the allosteric activation of the exchange by internal H^+ (Fig. 5) and (2) abolished growth factor activation of the exchanger. These findings support the view that NHE1 can be separated into two distinct functional domains: the transporter domain that has all the features required to catalyze amiloride-sensitive Na^+/H^+ exchange with a built-in H^+-modifier site and the cytoplasmic regulatory domain which determines the set point value of the exchanger (Wakabayashi et al. 1991).

Similar results have been obtained in other exchangers. In erythrocyte anion exchanger, Kopito et al. (1989) have shown that the deletion of the large NH_2-terminal hydrophilic cytoplasmic portion can retain the anion exchange activity. The author suggested that the NH_2-terminal domain is involved in the regulation of the exchange activity or in the structural organization of the cytoskeleton. Recent studies have indicated that limited pro-

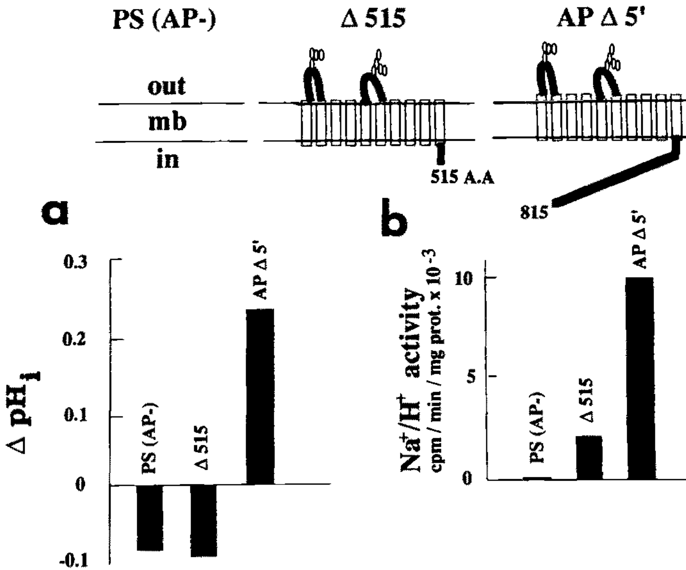

Fig. 4a, b. Functional expression of the wild-type and deleted Na^+/H^+ exchangers. The exchanger-deficient mutant cell line PS120 was stably transfected with human Ha^+/H^+ exchanger cDNA coding for the complete protein (wt) (815 amino acids), or coding only for the NH_2-terminal transmembrane domain (*Δ515*) (see Fig. 2). **a** Growth-arrested PS120 cells which were not transfected (*PS*) or transfected with *Δ* 515 and wt cDNAs were stimulated by growth factors (10 n*M* thrombin and 10 μg/ml insulin), and the change in the intracellular pH was measured. **b** Na^+/H^+ exchange activity was evaluated by measuring amiloride-sensitive $^{22}Na^+$ uptake. It is noted that deletion of the complete cytoplasmic domain preserves the activity but abolishes the growth factor activation. *out:* extracellular space; *mb:* membrane; *in:* intracellular space

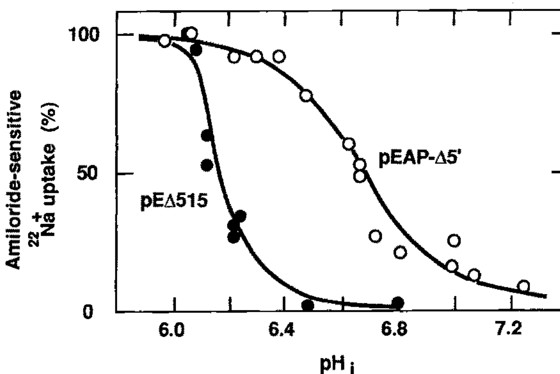

Fig. 5. Intracellular pH dependense of amiloride-sensitive $^{22}Na^+$ uptake. The PS120 cells were stably transfected with the wild-type (○) or the delection mutant (●) cDNA constructs. A change in pH_i was produced by loading the cells with different concentrations of NH_4^+ and washing the cells rapidly with a NH_4^+-free medium. It is noted that complete removal of the cytoplasmic domain shifts the pK value for intracellular H^+ to an acidic range by more than 0.5 pH unit

teolysis of the cardiac Na^+/Ca^{2+} exchanger with chymotrypsin eliminates the regulatory sites for cytosolic Ca^{2+} and ATP, leaving the exchanger in a state of high turnover rates (Hilgemann 1990). Chymotrypsin has been demonstrated to remove a fragment of relative molecular mass 50 kDa from the cardiac Na^{2+}/Ca^{2+} exchanger without loss of activity (Philipson et al. 1988). An analysis of the organization of a variety of transporter proteins, for example, plasma membrane H^+-ATPase (Portillo et al. 1989; Palmgren et al. 1990), Ca^{2+}-pump (Zvaritch et al. 1990), and Na^+/K^+-ATPase (Karlish et al. 1990), has also been carried out. Surprisingly, extensive treatment of the Na^+/K^+-ATPase with trypsin which results in the production of a 19 kDa fragment which is retained in the membrane could possess the ability to bind and transport Rb^+ (Karlish et al. 1990). These studies may support a common feature for many ion transport proteins; a limited number of transmembrane helices would be required for the ion translocation, and this transport would be regulated via one or more cytoplasmic hydrophilic domains. In the case of NHE1 and other isoforms two transmembrane domains, M5a and M5b (Fig. 2), are believed to play this crucial role.

3.2 Amiloride Binding Site

The potent inhibitor of the Na^+/H^+ antiporter, amiloride, consists of a substituted pyrazine ring with two amino groups attached at ring positions 3 and 5, a chloride moiety at ring position 6, and an acylguanidium group at ring position 2 (Fig. 6; see Benos 1988 for review). As amiloride is a weak base with a pKa of 8.7; it exists essentially as a monovalent cation in the physiological neutral pH range. Protonation occurs on the guanidine moiety of the molecule. This positive charge of the guanidinium group may partly account for the competitive behavior of amiloride with Na^+, Li^+, and H^+ and for the fact that amiloride inhibits other Na^+ transport proteins such as the Na^+-channel, the Na^+/Ca^{2+} exchanger and the Na^+-dependent amino acid transport (Grinstein et al. 1989). The inhibi-

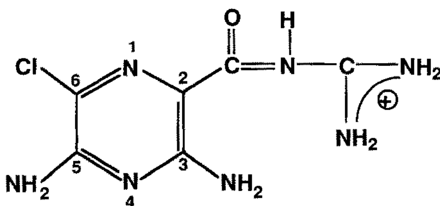

Fig. 6. Structure of protonated amiloride

tion constant (IC_{50}) of amiloride for the Na^+/H^+ antiporter is about 3 μM under sodium-free conditions. Various amiloride analogues, which are more potent than amiloride, have been synthesized and used for studies of Na^+/H^+ exchange. Alkylation of the 5-amino group of amiloride produces the most potent inhibition (L'Allemain et al. 1984; Zhuang et al. 1984).

There is substantial evidence that amiloride and its derivatives bind to the antiporter from the extracellular side of the exchanger; indeed, amiloride is a pure competitive inhibitor of the Na^+/H^+ antiporter with respect to external Na^+ in many types of cells (Benos 1988 for review), amiloride entrapped in the inside of dog red cell ghosts does not efficiently inhibit Na^+/H^+ exchange (Grinstein and Smith 1987), and complete removal of the C-terminal cytoplasmic domain (300 amino acids) of the antiporter preserves the amiloride-sensitive Na^+/H^+ exchange without significant change in the affinity for amiloride and MPA (Wakabayashi et al. 1991). This sidedness of amiloride binding must be due to the remarkable asymmetry of the topological structure of the exchanger (see Fig. 2). One might expect that the extracellular oligosaccharides are required for amiloride binding. However, the nonglycosylated antiporter expressed in insect cells (Sf9 cells) has an affinity for MPA which is comparable to the glycosylated exchanger expressed in the fibroblasts, suggesting that N-linked oligosaccharides are not required for amiloride binding (Fafournoux et al. 1991).

Furthermore, there is indirect evidence that amiloride has two distinct attachment or contact sites: (1) alkylation of the 5-amino group of amiloride greatly increases the inhibitory potency (L'Allemain et al. 1984; Zhuang et al. 1984), suggesting that there is a hydrophobic moiety required for the attachment of alkylated 5-amino group, in addition to the charged guanidinium group that could bind to the Na^+ site on the exchanger; (2) some of the exchanger mutants which have decreased affinity for amiloride analogues oppositely exhibit increased affinity for Na^+, suggesting an involvement of distinct sites (Franchi et al. 1986a) and (3) in lymphocytes (Dixon et al. 1987) and renal membrane vesicles (Ives et al. 1983), noncompetitive or mixed-type inhibition between Na^+ and amiloride or its analogues has been observed. It is possible that the charged guanidinium group of amiloride first enters the exchanger molecule from the extracellular side and its 5-amino moiety is fixed to the hydrophobic binding pocket near the extracellular surface if we assume that the competitive Na^+ binding site is located in the center of several transmembrane helices.

A possible strategy to assign the location of the amiloride binding site is to use radioactive and photolabile amiloride analogues. So far, several radioactive amiloride-related compounds have been used to identify the ex-

changer molecule. However, such a chemical technique is very difficult to apply to a protein of low abundance such as the Na^+/H^+ exchanger and with a ligand extremely hydrophobic. Another approach developed in our laboratory by Counillon et al. (in preparation) adopted the combination of a genetic approach and cDNA mutagenesis and consisted of the following steps; (1) selection of mutants cells expressing the exchanger resistant to amiloride or its derivatives, (2) isolation of cDNA clones coding for these amiloride-resistant exchanger from the mutant cells, (3) comparison of the amino acid sequence of the amiloride-resistant mutant deduced from cDNA sequence with that of the wild-type, and (4) determination of the essential region for amiloride binding by measuring the amiloride affinity of various chimeras between the amiloride-resistant and sensitive molecules. Using the approaches, a cDNA coding for the mutant exchanger has been cloned from the mutant cell line (AR300) which expresses an exchanger with 30 times less affinity for MPA than the wild type (Franchi et al. 1986a). Exchanger-deficient cells stably transfected with this mutant cDNA plasmid expressed an Na^+/H^+ exchanger with reduced affinity for MPA similar to the original MPA-resistant mutant cell line AR300. Furthermore, preliminary experiments using chimera mutant cDNAs between the amiloride-resistant and sensitive molecules revealed that the region between the second and the seventh transmembrane spanning segments determines the affinity for MPA (Counillon et al., in preparation).

Further information concerning the amiloride binding site will be obtained from the amino acid sequence of amiloride analogue-resistant isoforms. The NHE2, which has been recently described in kidney cell line (LLC-PK_1 cells), has 300-fold less affinity for ethylisopropylamiloride (EIPA) than NHE1 (Haggerty et al. 1988). Another type of isoform which was recently described in hippocampal neurons is totally insensitive to amiloride and its 5-amino-substituted analogues (Raley-Susman et al. 1991). Molecular regions of the amiloride-sensitive NHE1 which are not conserved in other isoforms resistant to amiloride and its analogues must be essential for the amiloride binding site. Subsequent work is required for the identification and complete determination of the amiloride-binding site.

3.3 Sodium and Proton Transport Sites

In bireactant reactions such as Na^+/H^+ exchange, we can principally assume two different reaction mechanisms, a ping-pong mechanism or a simultaneous one. In the former, after binding of the first substrate (e.g., ex-

tracellular Na^+), the first product (e.g., internal Na^+) is released before binding of the second substrate (e.g., internal H^+). In this case, the existence of only a single ion transport site is sufficient for binding of either Na^+ or H^+. In the latter mechanism, both substrates bind before the products are released. This case requires two distinct binding sites at the same time. Recent growing kinetic and mechanistic evidence supports the ping-pong reaction for describing the ion transport mechanism of many transporters which include Na^+, K^+-ATPase (Glynn and Karlish 1990 for review), gastric H^+, K^+-ATPase (Rabon and Renben 1990 for review), red cell anion transporter (Jennings 1989 for review), and Na^+/Ca^{2+} exchanger (Khananshivili 1990; Niggli and Lederer 1991).

In the Na^+/H^+ exchanger, steady state kinetic studies revealed that Na^+, Li^+ and H^+ are mutually competitive at least at the extracellular face of the exchanger with the exception of a few reports, suggesting the existence of a common binding site for these ions (Aronson 1985 for review). Recently transient kinetic studies of the Na^+/H^+ exchange in renal membrane vesicles has allowed the separation of the overall exchange reaction into several consecutive intermediary steps. An intravesicular-negative membrane potential enhances the amplitude of the transient initial burst phase during the initial single turnover, which probably corresponds to the Na^+ translocation step into the vesicles, suggesting the accumulation of positive charge in this step (Otsu et al. 1989) These accumulated data for various ion exchangers, though not conclusive, support the ping-pong mechanism for Na^+/H^+ exchange, in which a single site reciprocates across the membrane.

The Na^+/H^+ antiporter, in addition to Na^+/H^+ exchange, can drive Na^+/Na^+, Li^+/H^+, or Li^+/Li^+ exchange (Mahnensmith and Aronson 1985; Aronson 1985). This interchangeability of sodium (or lithium) and protons has been reported in many biological systems. In certain conditions, the Na^+/K^+-ATPase and gastric H^+/K^+-ATPase, instead of exchanging physiological ligands, can drive a slow H^+/K^+ and Na^+/K^+ exchange, respectively (Glynn and Karlish 1990; Rabon and Renben 1990). Interchangeability of Na^+ and H^+ has been also observed in the F_1F_0-ATPase in *Vibrio alginolyticus,* intestinal Na^+/glucose transporter, and *E. coli* H^+/melibiose cotransporter (Boyer 1988 for review). Boyer (1988) recently pointed out the possibility that in proton transport systems, proton translocation may occur by an initial complexation of the hydronium ion (hydrated proton, H_3O^+) with appropriately placed oxygens, such as the crown ether where proton binds as a hydronium ion in a manner analogous to Na^+. This concept may be also applicable to the Na^+/H^+ antiporter. However, there is also another distinct concept for proton translocation involving the participation of hydrogen-bonded chains of

protonatable groups, as hypothesized in *E. coli* H^+/lactose cotransporter (Kaback 1988) and bacteriorhodopsin (see Caspar 1990 for short review).

Our deletion mutant study suggested that Na^+ or H^+ directly binds to the N-terminal domain (500 amino acids) without involvement of the C-terminal cytoplasmic domain. In fact, within the transmembrane spanning segments, there are a limited number of oxygen-containing residues well conserved among the isoforms of different animal species, which may form the Na^+ and H^+ binding sites. Asp-159, 172 and Glu-346, 391 may be good candidates. Indeed, carboxylate-reactive reagents, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (Burnham et al. 1982) and *N,N'*-dicyclohexylcarbodiimide (DCCD; Igarashi and Aronson 1987; Kinsella et al. 1987) were shown to inhibit rat renal brush border membrane Na^+/H^+ exchange. The inhibition by the hydrophobic DCCD was protected by Na^+/Li^+ amiloride, suggesting that glutamic and aspartic acids within the transmembrane segments may be good candidates for ion-binding sites. A histidine-reactive reagent, diethylpyrocarbonate, was also shown to inhibit Na^+/H^+ exchange in both isolated brush border membrane (Grillo and Aronson 1986) and in thymic lymphocytes (Grinstein et al. 1985), suggesting that the imidazolium ring of histidine is the protonatable group which binds H^+ at the external transport site. However, such a group is not expected to bind Na^+. We are now trying to get a transport-deficient point mutant by the site-directed mutagenesis of amino acids expected to be involved in the ion translocation.

3.4 Proton Modifier Site

The Na^+/H^+ exchange process is fundamentally reversible and can operate in either direction. However, in terms of H^+ concentration dependence there exists an obvious asymmetry. In sharp contrast to the simple inhibition kinetics of external H^+, in that only one H^+ is involved, the concentration dependence of internal (intracellular) H^+ is much steeper with a Hill constant of more than 2. Such a steep pH_i dependence, which cannot be expected for one-to-one exchange, can be explained by assuming additional H^+ binding sites that are different from the H^+ transport site. Indeed, Aronson et al. (1982) have observed that H^+ loaded into brush border membrane vesicles stimulated Na^+ efflux from the vesicles, an effect opposite to that expected from pure competition for the internally oriented transport site. From these findings, they proposed the existence of internal H^+ modifier sites at which H^+ binding turns on the operation of Na^+/H^+ exchange.

Deletion mutant studies revealed that complete removal of the C-terminal cytoplasmic domain of the exchanger shifted the pK value by 0.5 pH unit or more to an acidic side, but preserved the allosteric activation by internal H^+ (Fig. 5; Wakabayashi et al. 1991). This finding implies that H^+-modifier site(s) are located within the N-terminal transmembrane domain, and the affinity for H^+ at this site is increased by the presence of the cytoplasmic domain. The pK value in the range of 6 to 7 implies the involvement of amino acid residues which are protonated in this pH range. The imidazole residue of histidine is a likely candidate. Several conserved histidine residues exist in the small cytoplasmic loops or in the transmembrane, spanning segments within the N-terminal domain which were predicted by the secondary structure model of NHE1 (see Fig. 2). For example, His-121, 349, 407 and 408 may be involved in the H^+-modifier site. Alternatively the glutamic acid residues conserved between M5a and M5b membrane segment are good candidates.

However, similar to intracellular H^+, intracellular Na^+ has been reported to activate the exchanger allosterically (Green et al. 1988a). In agreement with the competitive behavior of Na^+ and H^+ at this activation site, osmotic stress modifies the affinity for intracellular Na^+ in a different direction from that for intracellular H^+ (Green et al. 1988b). Therefore, the observations that Na^+ appears to compete with H^+ at this H^+-modifier site may exclude the view that H^+ binding at this site reflects protonation of the protonatable amino acids of the exchanger.

4 Phosphorylation and Regulation

4.1 Enhanced Phosphorylation in Response to Mitogens

The molecular mechanism of the exchanger activation in response to extracellular signals is not known. It has been hypothesized that phosphorylation of either the antiporter itself or of an ancillary protein may be responsible for the alteration of the exchange activity (Grinstein 1988 for review). The initial signaling pathway which leads to the mitogen activation of the Na^+/H^+ exchanger (NHE1) can be grouped into two categories: (1) receptors with an intrinsic tyrosine kinase activity and (2) G protein coupled receptors.

In the former type of receptors such as those for epidermal growth factors (EGF), insulin, insulin-like growth factor (IGF-1), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and colony-stimulating factor (CSF-1) receptors, the initial event following ligand binding is

the stimulation of receptor tyrosine kinase activity (Ullrich and Schlessinger 1990 for review). On the other hand, in the latter type of receptors, the initial ligand binding triggers several different signaling pathways, depending on the types of G protein coupled to the receptor (Pouysségur 1989; Birnbaumer et al. 1990). Binding of growth factors such as α-thrombin, vasopressin, bombesin, serotonin and α1-adrenergic agonists to their respective transmembrane receptors stimulates a G protein which interacts with membrane-bound phospholipase C. The activated phospholipase C catalyzes the hydrolysis of phosphatidylinositol 4,5 bisphosphate (PIP2) into diacylglycerol (DAG) and inositol 1,4,5 trisphosphate (IP3). The DAG is a natural activator of protein kinase C, while IP3 triggers Ca^{2+} release from internal Ca^{2+} stores, which in turn activates various Ca^{2+} binding proteins such as calmodulin-dependent kinase (Berridge 1987 for review). Binding of ligands to the receptors coupled to another member of the G-protein family, for example, β-adrenergic and postaglandin E receptors, stimulates the membrane-bound adenylate cyclase. The activated cyclase increases the cellular cAMP concentration, which leads to the activation of cAMP-dependent kinase.

A large number of publications have suggested that the kinases described above (receptor tyrosine kinases, protein kinase C, protein kinase A and Ca^{2+}/calmodulin kinase) are involved in the modulation of the antiporter activity. However, we have to say that there are a lot of contradictory results (see Sardet et al. 1991 b for review). One reason for these contradictory results may be due to the different isoforms expressed in the different cell types. It is well accepted that protein kinase C is involved in activation of NHE1; DAG and phorbol esters activate the exchange activity (Moolenar et al. 1984; Paris and Pouysségur 1984), putative inhibitors of protein kinase C block the exchange (Lowe et al. 1990); and phorbol esters fail to activate the exchanger on protein kinase C-depleted cells (Huang et al. 1987). In contrast, activation of protein kinase C has been reported to inhibit the apical form of antiporter (putatively NHE2) in epithelial cells from small intestine and kidney (Rood et al. 1988).

The effect of intracellular Ca^{2+} on the Na^+/H^+ exchange activity is still open to debate. The direct elevation of intracellular Ca^{2+} concentration by calcium ionophores has been reported to activate the exchange activity in WS-fibroblasts (Hendey et al. 1989), rat parotid acinar cells (Manganel and Turner 1990), and in human platelets (Kimura 1990), and to fail to produce this effect on 3T3 cells (Ives and Daniel 1987), HF human fibroblasts (Moolenaar et al. 1983), smooth muscle cells (Huang et al. 1987), and hamster lung fibroblast (CCL39; Pouysségur et al., unpublished observations). On the other hand, Ca^{2+}/calmodulin has been shown to inhibit the exchange activity on ileal brush border membrane ves-

icles (Emmer et al. 1989). In addition, in this preparation, an increase in intracellular cAMP concentration has been reported to inhibit the exchange activity (Kahn et al. 1985).

In a fibroblast cell line ER22 which was derived from Chinese hamster lung fibroblast, EGF and thrombin (THR) have an equal potency to acti-

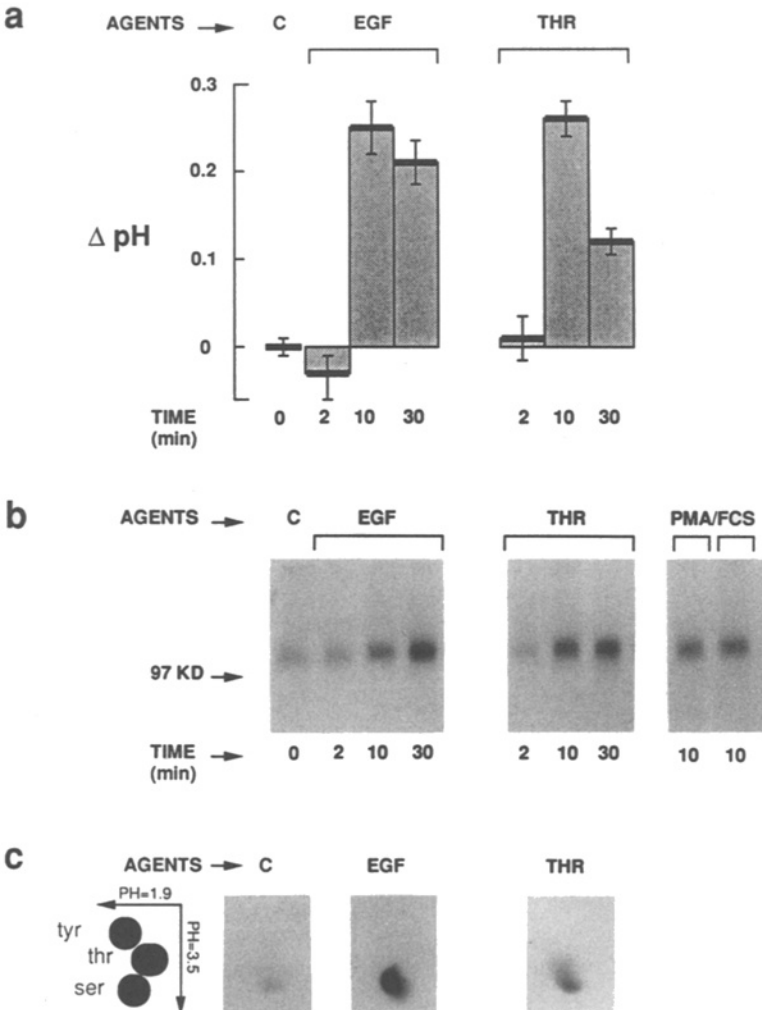

Fig. 7a–c. Parallel increase in the phosphorylation of serine residues and the Na^+/H^+ exchange activity. Quiescent ER22 cells were stimulated by either 40 n*M EGF*, 10 n*M* thrombin (*TRH*), 100 ng/ml phorbol dibutyrate (*PMA*) or 10% fetal calf serum (*FCS*). The intracellular pH increases (**a**) and in vivo phosphorylation (**b**) were measured on parallel cultures and under the same conditions of stimulations. Phospho-amino acid analysis (**c**) was performed under the same conditions of 30-min stimulation with thrombin. The mitogens used enhanced the phosphorylation state of the antiporter exclusively on serine residues

vate the Na^+/H^+ exchange (L'Allemain et al. 1989; Sardet et al. 1990). In this cell line, these two growth factors use clearly separate signaling pathways, being coupled to a receptor tyrosine kinase and a G protein-coupled receptor, respectively (Chambard et al. 1987; L'Allemain et al. 1989). The activation of the latter receptor, but not the former, leads to protein kinase C activation and a transient intracellular Ca^{2+} increase in ER22 cells. To elucidate how these distinct signals activate the exchanger, we examined the ability of these growth factors to alter the phosphorylation state of the exchanger using specific antibodies raised against the cytoplasmic domain. In either growing or growth-arrested ER22 cells, the antibody immunoprecipitated a single glycosylated phosphoprotein of 105–110 kDa labelled with ^{32}P (see Fig. 8). Therefore, the antiporter is a phosphoglycoprotein. Phosphorylated antiporter has been demonstrated in several cell types: Chinese hamster lung fibroblast (CCL39, ER22; Sardet et al. 1990), human carcinoma cells (A431; Sardet et al. 1990), mouse embryonic EC cells (P19; unpublished results), and human platelets (Livne et al. 1991).

Mitogenic stimulation by either EGF or thrombin increased the phosphorylation state of the exchanger in ER22 cells, temporally in parallel with the stimulation of the exchange activity (Fig. 7). This is the first support for the hypothesis that the exchange activity is controlled by the phosphorylation state of the antiporter protein itself. Furthermore, the stimulation of the phosphorylation occurred exclusively on serine residues (Fig. 7) and on the same phosphopeptides (Fig. 8), regardless of the different types of signals. These results suggest that there is an early integra-

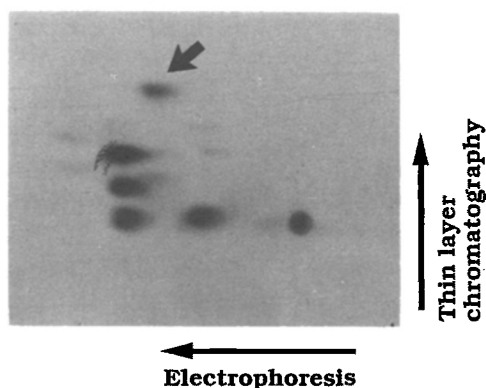

Fig. 8. Tryptic phosphorylation map of the human Na^+/H^+ exchanger. The cells stably expressing the exchanger were labeled with [^{32}P]orthophosphate and subsequently immunoprecipitated with the specific polyclonal antibody. Trypsin digestion of the immunoprecipitated exchanger was analyzed by two-dimensional thin-layer electrophoresis/chromatography. In response to mitogenic stimulation (EGF or thrombin), this map shows the occurrence of a new phosphorylation site (*arrow*)

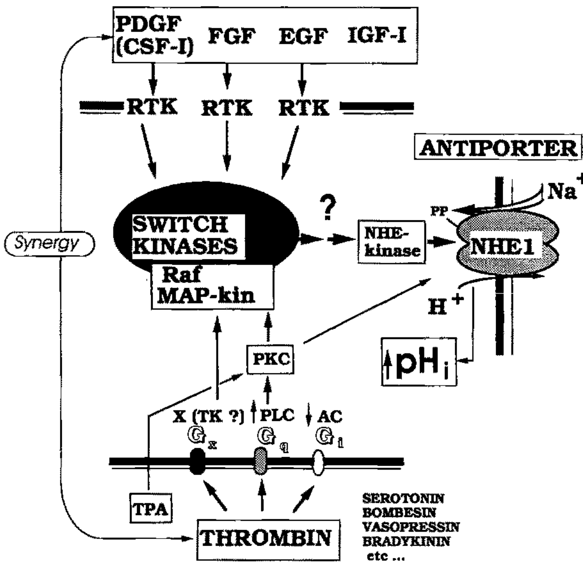

Fig. 9. Growth-signaling pathways and Na^+/H^+ antiport activation. This scheme outlines the hypothetical mitogenic pathways leading to activation of the Na^+/H^+ exchanger in CCL39 cells and subsequent rise in cytoplasmic pH. Growth factors such as *EGF, PDGF* or *FGF* activate a transmembrane receptor-coupled tyrosine kinase (*RTK*). A second family of growth promoting agents acting through G protein-coupled receptors which are capable of either inhibiting or stimulating adenyl cyclase (*AC*) or activating polyphosphoinositide-specific phospholipase C (*PLC*), and as a working hypothesis by activating *X*, other unknown effectors. As illustrated, protein kinase C (*PKC*) also activated by phorbol esters (*TPA*) does not represent the only way to activate the exchanger. The switch kinase, like MAP-kinase (*MAP-kin*) might serve to integrate various extracellular signals leading to activation of a specific *NHE1*-kinase.

tion which inputs the signals from distinct signaling pathways and outputs a common effect, exchanger activation. An attractive hypothesis is that this integrator is a switch kinase like Mitogen Activated Protein (MAP) kinase (Rossomando et al. 1989) that will in turn activate kinase-kinases, activating at the end of the cascade-specific serine kinases such as S6 kinase (Ballou et al. 1988) and NHE1-kinase (Fig. 9).

Another strong piece of evidence to suggest the relationship between protein phosphorylation and Na^+/H^+ exchanger activity comes from the use of okadaic acid on living cells. It has been demonstrated by in vitro studies that okadaic acid is a potent membrane-permeable inhibitor of protein phosphatases 1 and 2A which are specific for serine/threonine phosphoryl residues (see Cohen et al. 1990 for review). The addition of okadaic acid to quiescent cells increased the phosphorylation of the exchanger on the same phosphopeptides as those enhanced by EGF or α-thrombin in

parallel with the exchanger activation (Sardet et al. 1991 a). These results reinforce the notion that the phosphorylation of the exchanger controls its set point value.

4.2 Phosphorylation-Dependent Coupling of the Cytoplasmic Domain with "H^+-Sensor"?

Which serine residues respresent the critical phosphorylation site(s) for the determination of the set point value and for growth factor activation? The recent progress in recognizing specific phosphorylation site motifs (Kemp and Pearson 1990) has allowed the definition of potential "consensus sites". Several of these sites (C-kinase, Ca^{2+}/calmodulin-dependent kinase II, for example) have been identified in the exchanger. The analysis of phosphopeptide tryptic maps has provided further important information (Sardet et al. 1991 a; Fafournoux et al. 1991). Tryptic peptides of *in vivo* ^{32}P-labeled Na^+/H^+ exchanger from ER22 cells treated with growth factors (EGF, THR), serum, or okadaic acid were analyzed by two-dimensional thin-layer electrophoresis/chromatography (Fig. 8). The phosphopeptide map of NHE1 suggests the existence of several sites of phosphorylation since the relative amount of phosphate incorporated differs in some peptides in the absence and presence of growth factors. It was noted that following mitogenic stimulation, a novel ^{32}P-labeled peptide could be detected (Fig. 8). The identical NHE1 phosphorylation pattern observed in EGF- and α-thrombin-stimulated cells suggested the existence of a specific NHE1-kinase.

Detailed studies suggest that the cytoplasmic region between amino acid 566 and 635 must be required for exchanger activation in response to signals such as PDGF, phorbol esters, and okadaic acid (Wakabayashi et al. 1991; see also Fig. 4). Therefore, it is likely that one or more serine residues located in the cytoplasmic region between amino acid 566 and 635 is an essential phosphorylation site(s) for mitogen activation. There are eight serine residues within this region of human Na^+/H^+ exchanger (see Fig. 2). However, mutation of each of these serine residues to alanine did not abolish growth factor activation (Wakabayashi et al., in preparation). An interesting finding is that a point mutation of serine 648 which is thought to be a good consensus serine residue for protein kinase C-mediated phosphorylation did not abolish mitogen activation, suggesting that protein kinase C does not activate the exchanger through direct phosphorylation at this serine residue. Several possibilities remain. First, phosphorylation at multiple serine residues within the cytoplasmic domain is required for mitogen activation. Second, a critical phosphorylation site is located within the N-terminal domain (500 amino acid). Work in this area is ongoing.

The NHE1 is constitutively phosphorylated in quiescent cells. What is the role of phosphorylation in nonstimulated cells? Cellular ATP depletion by incubation with metabolic inhibitors has been shown to reversibly inhibit Na^+/H^+ exchange activity in A431 carcinoma cells (Cassel et al. 1986), in spontaneously beating cardiac cells (Weissberg et al. 1988), in rat aortic smooth muscle cells (Little et al. 1988), and in PS120 fibroblast cells transfected with human antiporter cDNA (Wakabayashi et al. 1991). In these cells, an inhibition of Na^+/H^+ exchange activity correlates with the extent of the reduction of the ATP pool. With the exception of the data obtained by Little et al. (1988), this inhibition of exchange activity is due to the decrease in the affinity of the exchanger for internal H^+. These experiments indicate that a physiological cellular ATP level is indispensable for the operation of the exchanger at a physiological pH_i range, although the Na^+/H^+ exchange reaction itself does not require the energy from ATP hydrolysis. In the PS120 fibroblast cell line transfected with human exchanger cDNA, cellular ATP depletion reversibly reduces the phosphorylation state of the antiporter, abolishes growth factor activation, and reduces the H^+-affinity of the H^+-sensor (Wakabayashi et al. 1991). Both the increased pH_i dependence of the exchanger and the lack of growth factor activation were mimicked by complete removal of the cytoplasmic domain. These results suggest that dephosphorylation at a critical site(s) induced by ATP-depletion must alter the conformation of the cytoplasmic domain so as to reduce the intracellular H^+ sensitivity. A working hypothesis has been proposed which states that mitogen activation occurs through the reversible phosphorylation-dependent "coupling" of this cytoplasmic domain with an internal H^+-modifier site (Wakabayashi et al. 1991).

Although the molecular basis for aforementioned "coupling" is not known, phosphorylation-dependent subunit-subunit interaction may be a key event as shown for glycogen phosphorylase (Sprang et al. 1988). Otherwise, the pK value of the protonatable residue(s) of the H^+-modifier site may be directly increased by the access of net negative charges introduced by the phosphorylation. Indeed, negative charges have been shown to be important for phosphorylation-dependent change in the function of some proteins. An amino acid change from threonine or serine to aspartic acid residues has been shown to mimic the phosphorylation of these phosphorylatable residues in Ca^{2+}/calmodulin kinase II (Soderling 1990 for review) or proto-oncogene c-*fos* protein (Ofir et al. 1990). In order to know the molecular mechanism of the phosphorylation-dependent interaction of the cytoplasmic domain with H^+ sensor, the critical phosphorylation site of the exchanger should be finally identified.

5 Conclusion

In this chapter, we have reviewed recent progress in the structure function studies of the Na^+/H^+ antiporter. However, to answer most of the questions put in the introduction, further work will be required. Molecular cloning and immunological techniques revealed the following interesting findings: There are at least two pharmacologically and spatially distinct isoforms of Na^+/H^+ exchanger, NHE1 and NHE2, which are derived from different genes; the NHE1 is an oligomeric phosphorylated glycoprotein with 815 amino acids; mitogenic stimulation of the exchanger by distinct types of external signals leads to the enhanced phosphorylation of NHE1 on serine residues and the same phosphopeptides; and the NHE1 can be separated into two distinct functional domains, the transporter domain which can catalyze amiloride-sensitive Na^+/H^+ exchange with a built-in H^+ sensor and the cytoplasmic regulatory domain that determines the set point value of the antiporter. From these data, we propose that activation of NHE1 in response to external stimuli occurs via a phosphorylation-induced conformational change of the regulatory domain, tentatively located in the large C-terminal cytoplasmic domain. Further work is required to identify precisely the phosphorylation and H^+ sensor sites and the kinase(s) involved. On the other hand, deletion mutant studies revealed another surprising finding that the last C-terminal 180 amino acids are not necessary both for the exchange activity and growth factor activation. What is the role of this large portion? A likely possibility is that this portion contacts with cytoskeleton, like erythrocyte anion exchanger (Low, 1986 for review). It is possible that the activation of Na^+/H^+ exchange by intracellular signals in response to osmotic stress (Grinstein et al. 1989) or cell spreading (Schwartz et al. 1989; Margolis et al. 1988) are transmitted via interaction of this regulatory domain with cytoskeletal elements. This hypothesis is currently under investigation.

Acknowledgments. We thank K. Suwen, E. Van Obberghen-Shilling, F. MacKenzie, P. Syme, and P. Pilch for fruitful discussion and critical reading of the manuscript, D. Grall for technical assistance on cell culture, and M. Valetti for manuscript preparation.

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Rev. Physiol. Biochem. Pharmacol., Vol. 119
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DNA Damage-Induced Gene Expression: Signal Transduction and Relation to Growth Factor Signaling

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1 Introduction

It has been recognized for some 30 years and well documented since that an organism adapts to the conditions of its habitat by adjusting both the program of genes expressed and the initiation rate or replication. The in-

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herited static complement of genetic information is made use of in a dynamic way. Key elements for these genetic control mechanisms are the components of the transcription, translation and replication machineries and specialized structures that perceive a change of condition to which the organism needs to react. Organisms perceive, for example, the presence of nutrients, signals from intercellular communication, light, and other environmental conditions. Much more recently it has been observed that organisms also react massively to conditions that are outside the "physiologic" range. Even severely toxic agents can elicit a dramatic change in gene expression and in replicative behavior. The genetic response to environmental adversities (stress response) is not one entity but rather a set of mechanistically overlapping reactions. As molecular understanding progresses these mechanisms can be discerned. Here, we review the current knowledge of the mammalian stress response with particular emphasis on the molecular mechanism of the so-called DNA damage response, that is, the response to treatment of mammalian cells with DNA damaging agents. For other aspects of the stress response the reader may consult one of the following articles: previous description of the DNA damage response by Herrlich et al. 1986; Mai et al. 1989; 1990; Kaina et al. 1989; Ronai et al. 1990; the response to phorbol esters by Karin and Herrlich 1989; Rahmsdorf and Herrlich 1990; the activation of human immunodeficiency virus expression and of other genes by sunlight and UV by Zmudzka and Beer 1990; Tyrrell and Keyse 1990; gene regulation by active oxygen species by Cerutti 1985; Colburn 1990; Storz et al. 1990; heat shock by Lindquist 1986; Sorger 1991; heavy metal induced gene expression and carcinogenesis by Furst and Radding 1984; Caltabiano et al. 1986; Shelton et al. 1986; Schäfer and Kägi 1991; Gebhart and Rossman 1991; the response to p450 inducing agents by Nebert and Gonzalez 1987; Gonzalez and Nebert 1990; DNA damage induced gene expression and the adaptive response in bacteria by Walker 1985; Ossanna et al. 1987; Vaughan et al. 1991; DNA damage induced gene expression in yeast by Jones and Prakash 1991; stress-induced gene expression in plants by Weisshaar et al. 1991.

2 Cellular Stress

According to the classical definition the term stress is reserved for the "organismic stress" (Selye 1966) which describes hormonally mediated biological phenomena elicited by adverse external influences. The classical definition stems from a period prior to cell biology. We now define stress response more broadly as all reactions of cells to adverse conditions. Adverse

external influences are exerted by agents whose quality or quantity is outside that "normally" encountered. (This description suggests a threshold. We will return to the threshold problem below.) For instance, reactive oxygen intermediates are physiological by products of metabolism. Increased levels or altered composition may, however, induce stress. For other stress agents, all dose ranges are considered adverse (e.g., X-rays). Temperatures below or above physiologic condition and sudden changes of temperature induce cellular stress. An interesting variety is represented by certain nonpolar plant metabolites and environmental pollutants that become stress agents as a result of metabolic conversion. The conversion intended at detoxification and excretion, produces water-soluble reactive intermediates which are genotoxic.

The genetic constitution permits cells to react to stress in only a limited number of ways. The regulatory components, transcription factors, replication factors, critical components of the translational and protein processing machinery have been adjusted in evolution for normal cellular functions. If a stress agent causes a genetic response, it must feed into these normal physiologic regulatory mechanisms, and one would expect that stress responses be fairly uniform, overlapping rather than agent-specific. This is what has been found. A small number of overlapping response patterns seem to coexist (Fig. 1). To decide between overlap or agent specifici-

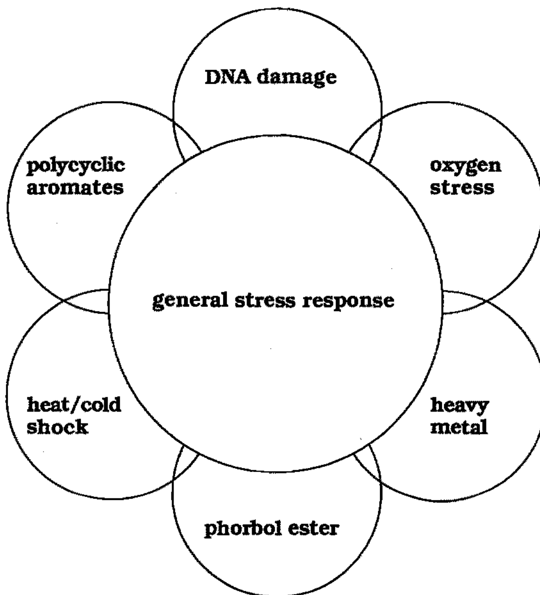

Fig. 1. Agent-specific and overlapping genetic programs

ty of a response, a careful study of different doses and knowledge of the molecular mechanism would be desirable. Different doses must be considered because most responses go through an optimum, with higher or lower doses erroneously suggesting nonresponsiveness to this particular agent.

Work on bacteria has revealed responses that can be classified according to a molecular mechanism. For instance, the SOS response (Radman 1974) includes everything that depends on the activity of a regulating gene, recA (see review by Walker 1985), and the adaptive response represents a specific regulatory mechanism involving the *ada* gene and its function (Lindahl et al. 1988; Vaughan et al. 1991). It is quite possible that, in the end, similar mechanisms will be detected in eukaryotes. However, prior to sufficient molecular understanding we prefer to pragmatically define responses according to the agent or class of agents eliciting the response (Fig. 1). If a gene is part of more than one context, this could either mean that different stress agents act on this gene by one mechanism or that the gene carries multiple modules that permit regulation by more than one stress agent.

3 DNA-Damaging Agents Induce Changes in the Abundance of Gene Products and Cause Overreplication

The analysis of the DNA damage-induced genetic response has been influenced by the prior and parallel investigations into serum, heat shock and phorbolesterdependent reactions. The changes observed after treating cells in culture with DNA-damaging agents indeed resemble those after growth factor or phorbol ester contact. Growth factors, phorbol esters, and DNA-damaging agents alike induce genes required for cell cycle progression (for a list of recent references see Rahmsdorf and Herrlich 1990). Unlike growth factors, DNA-damaging agents induce replication at an inappropriate time with respect to the normal cell cycle. In synchronized human diploid fibroblasts, however, several DNA damaging agents induce bursts of replicative synthesis similar to the one seen after growth factor treatment (Cohn et al. 1984). The replication can be quite excessive, suggesting persisting stimulation of the replicative machinery.

Following the course of action in time of a DNA-damaging agent several levels of the response can be defined and explored separately.

1. The presence of the DNA damaging agent is perceived such that a stress response is initiated.
2. The primary target is connected to critical parts of the normal cellular machinery for expression and replication (signal transduction).

3. These critical parts are preformed limiting protein components that upon signal transduction change their behavior, for example, by posttranslational modification, and that subsequently initiate the genetic response. Depending on the type of process influenced by the DNA damaging agent, these limiting proteins will be replication factors, transcription factors, spliceosome components, enzymes that process RNA or protein, or regulatory proteins of translation. (We have recently reviewed the target molecules addressed by phorbol esters: Rahmsdorf and Herrlich 1990.)
4. Extinction of the stress-induced signals: The transient nature of the stress response is ascertained at various levels.
5. As a result of the initial response, secondary and further steps will be induced. For instance, overreplication of DNA sequences will form an obstacle to cell cycle progression and be the substrate for excision and reintegration into the linear order of DNA. Thus the net result will be insertional mutagenesis (Lücke-Huhle et al. 1990). Induced gene products may themselves be transcription factors and cause secondary rounds of gene expression which in turn alter the cellular phenotype.

4 Perception of the Presence of the DNA-Damaging Agent

Obviously, the presence of a stimulator must be perceived by the cell. Specific growth factor receptors bind the growth factor and, in consequence, exert an altered behavior, e.g. an enzymatic activity or an altered interaction with another membrane protein (Williams 1989). The presence of phorbol esters is registered by a class of cytoplasmic protein kinases (Nishizuka 1988; Gschwendt et al. 1991) and heat shock is sensed directly by specialized transcription factors (Craig and Gross 1991). Accordingly, the presence of a DNA-damaging agent must be recognized and transformed to the "currency" understood by the cellular machinery.

The primary interaction could theoretically occur in a number of ways (Fig. 2). To state the extreme possibilities first: The agent could directly interact with DNA and cause DNA damage which could then be the necessary intermediate for dependent reactions. Or the agent could elicit a cellular response by a mode independent of its DNA-damaging action. The agent would cause DNA damage, but the interaction eliciting the genetic response would be elsewhere (see below for the action of benzpyrene and TCCD).

The largest class of regulatory proteins appears to be activated in response to DNA damage with DNA damage as a necessary intermediate.

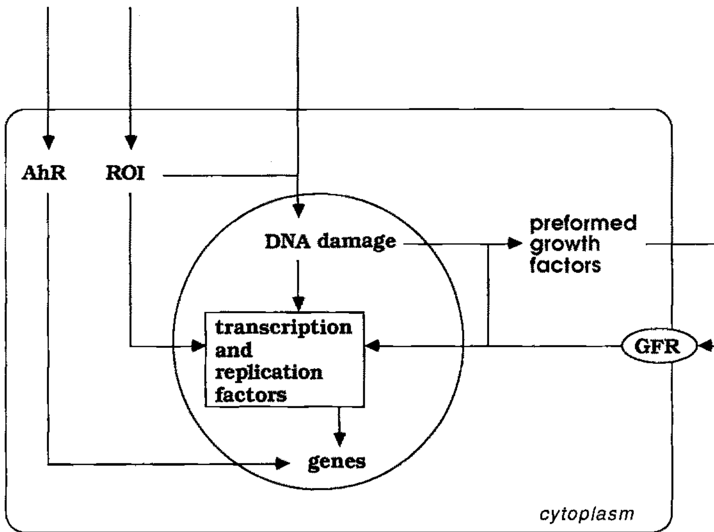

Fig. 2. Gene activation by DNA damaging agents. Note that the gene products induced may convert agents to carcinogens. *ROI*, reactive oxygen intermediates; *GFR*, growth factor receptor; *AhR*, aromate receptor

The hypothesis envisages the generation of a signal chain elicited by the altered DNA structure. DNA damage as a necessary intermediate for the induced expression of genes and for the overreplication from viral origins has been documented by (1) the wavelength of UV maximally effective in the induction of the stress response (Ronai et al. 1987; Yakobson et al. 1989; Stein et al. 1989a), (2) the fact that specific DNA-repair enzymes can remove the signal-eliciting damage and suppress the stress response by these agents (Stein et al. 1989a) and (3) the induction of the stress response by the introduction of damaged DNA into nontreated cells (preliminary results together with S. Mai, S. Lavi et al.).

The DNA-damaging agents may cause DNA damage and, in this process or independently, undergo another decisive interaction. An example to this point is the possible generation of a "second messenger" which then influences regulatory proteins directly or through a signal transduction chain. Several DNA damaging agents, e.g., ionizing radiation, generate reactive oxygen intermediates (Repine et al. 1981; Fig. 2). Reactive oxygen intermediates are also formed as a result of the cellular metabolism (Simic et al. 1989; Joenje 1989; Cadenas 1989). Scavenging mechanisms exist that strictly control the level of these compounds (Joenje 1989; Cadenas 1989). It is not totally clear which quality of the reactive oxygen intermediates

would determine a stress response. H_2O_2, ionizing radiation, long-wave UV and tumor necrosis factor-α (TNF-α) may influence the level or composition of such intermediates (Repine et al. 1981; Klebanoff et al. 1986). There are also reports on the generation of oxygen intermediates following phorbol ester treatment of certain cell lines (Gennaro et al. 1986; Christiansen 1988; Hanson and DeLeo 1989; Applegate et al. 1991). It has been postulated that regulatory proteins (such as transcription factors) could directly sense radical attack or redox changes. Particularly NFκB and Fos/Jan could be such targets. Hydroxyl radical savengers inhibit lymphocyte mitogenesis and NFκB activation (Novogradsky et al. 1982; Chaudhri et al. 1986; Staal et al. 1990; Schreck et al. 1991). Fos in contrast, is inactivated by oxidation in vitro (Abate et al. 1990).

The DNA-damaging agent, if a chemical, could bind directly to the regulatory component, thereby activating it. The only example yet documented is that of "indirectly acting" carcinogens such as benzpyrene and TCCD (dioxin). A family of aromatic hydrocarbon receptor proteins appears to be activated by the ligand, similarly to steroid receptors, and function in the nucleus as transcription factors (Denison et al. 1986; Cuthill et al. 1987; Perdew and Poland 1988). As a result of the induced transcription the agent is metabolized. One of the metabolic products causes DNA damage. Thus this class of agents may trigger two types of response: one directly through the hydrocarbon receptor, one as a consequence of DNA damage.

5 Signal Transduction

That the primary site of interaction between agent and cell, and the responding genetic structure are two separate entities and that these entities require a process of signal transduction was first recognized by cell fusion experiments. In the heterokaryon produced by the cell fusion between a treated and a nontreated cell, the nucleus (or other components) of the treated (e.g., irradiated) cell signals the other (e.g., nonirradiated) nucleus. Its response is detected by an indicator gene (Nomura and Oishi 1984; van der Lubbe et al. 1986; Lambert et al. 1986; Lücke-Huhle and Herrlich 1987). This type of experiment shows at the same time that the signaling involves the cytoplasm and presumably the whole cell.

The analysis of signal transduction in general, whether growth factor or phorbol ester or DNA damage dependent, is still rudimentary. Essentially, only the signal generator and the receiving ends are known. The following describes what is known for the DNA damage-induced signal chain.

5.1 Which Molecules Monitor the Damage to DNA?

The proteins that recognize damaged DNA and subsequently emit a "signal" are as yet unknown. By band shift experiments proteins have been detected that bind to damaged DNA, e.g., to UV-damaged DNA (Chu and Chang 1988; Patterson and Chu 1989; Hirschfeld et al. 1990; Chao et al. 1991); to X-ray-damaged DNA (Boothman et al. 1990); to *cis*-platinum-DNA adducts (Toney et al. 1989; Chao et al. 1991); and to DNA single-strand-breaks (Poly (ADP-ribose) polymerase, Cleaver and Morgan 1991). These proteins could represent signal-generating molecules but could also simply be part of repair reactions and be irrelevant for signal generation. Poly (ADP-ribose) polymerase modifies proteins, but functional alteration of a regulatory protein by polyADP ribosylation has not yet been documented. Recently DNA-dependent protein kinases have been discovered (Jackson et al. 1990; Lees-Miller et al. 1990; Carter et al. 1990). It will be interesting to see whether such protein kinases can be activated by DNA damage. Interestingly, protein kinase inhibitors block the damage-induced gene expression (Büscher et al. 1988; Stein et al. 1988; Krämer et al. 1990).

Prior to knowledge of the signal-eliciting primary proteins recognizing DNA damage, the question of damage specificity cannot be conclusively resolved. Two possibilities can be envisaged: either different types of DNA damage (cyclobutane pyrimidine dimers, 6-4 photoproducts, single-strand breaks, O^6-methyl G etc.) are recognized by different proteins which then generate presumably one and the same signal. To hypothesize with a minimal number of such damage-binding molecules, repair enzymes could be signaling proteins at the same time. Or, alternatively, a common property of DNA damage is recognized. Signaling could then be the function of proteins that recognize one or a few general features of altered DNA structure (e.g., a kink of helix, single strandedness).

If each signal generator stimulated a different signal transduction pathway and led to modification of a different target protein, e.g., transcription factor, the analysis of these regulatory proteins could provide clues on both the signal generator and the transduction pathway. Evidence, however, exists for the activation of seemingly one and the same regulatory component (e.g., the limiting component in the SV40 origin binding activity; Lücke-Huhle et al. 1989; Mai et al. 1990; the transcription factors AP-1 and $p67^{SRF}$; Büscher et al. 1988; Stein et al. 1989a) by several agents, e.g., alpha, X-ray, *N*-methyl-*N*-nitro-*N*'-nitrosoguanidine (MNNG) and UV. This suggests convergence of signal transduction pathways if not identity. Whether the modification of a given factor upon stimulation by different agents is indeed identical, needs to be analyzed in more detail. An example will be discussed below.

A point for lesion-specific induction has been brought forward by the analysis of a larger collection of damage-inducible cDNA clones. A subclass of these genes is only induced by UV-C, but not by methylmethane sulfonate (MMS; Fornace et al. 1988a). The mechanisms of activation of these genes have not yet been investigated.

5.2 The Possibility of a Growth Factor Loop

One could argue that an intermediate in the signal transduction between DNA damage and transcription factor activation might be the release of preformed growth factors that intracellularly or outside the plasma membrane act on their specific receptors (Fig. 2). These then induce the signal transduction mechanism to the nucleus. This suggestion sounds unlikely in view of the fast kinetics of the stress response (the replication origin binding activity is maximal within less than 30 min, Lücke-Huhle et al. 1989; early response genes are activated within 5 – 10 min, for references see Rahmsdorf and Herrlich 1990). However, the release of growth-factor-like proteins in response to UV irradiation has been detected (Schorpp et al. 1984; Ansel et al. 1984; Rotem et al. 1987; Maher et al. 1988; Stein et al. 1989b). Thus the induction of gene expression through a growth factor loop seems possible, at least for the slower portions of the response. The factors from the supernatants of UV-treated cells have been identified with respect to their collagenase-inducing activity (Krämer et al. unpublished). Interleukin 1α (bFGF) and basic fibroblast growth factor (Il1α) account for all such activity present in conditioned medium from HeLa cells. Both Il1α and bFGF mRNA levels and protein syntheses are elevated after UV treatment. Thus, these factors could act back onto the cells producing them and cause a prolonged secondary stress response.

In an intriguing experiment (Krämer et al. unpublished) we found recently that the UV-induced transcription of c-*fos* (a matter of minutes and independent of new protein synthesis) can be blocked by suramin, a drug that is supposed to prevent growth factor-receptor interactions (Garrett et al. 1984; Betsholtz et al. 1986; Coffey et al. 1987; Huang and Huang 1988). Phorbol ester-dependent c-*fos* expression was not influenced, ruling out a nonspecific interference with gene expression. Our experiment suggests a UV-induced release of preformed growth factors as an intermediate of the signal transduction mechanism. One would need to postulate a DNA damage-dependent signal that is received by the preformed growth factors or their "storage structure". They would then be released, activate specific receptors, and trigger signal chains that end at the transcription factors mentioned previously (Fig. 2). The growth factor-receptor interaction would

occur at the plasma membrane or intracellularly (suramin interferes also with growth factor-growth factor receptor interactions that are not accessible by antibody from outside the cell, Yayon and Klagsbrun 1990). The whole process would still be cycloheximide-resistant as it does not involve new protein synthesis. If the growth factor loop indeed exists, antisense or recombinational knock-out experiments of Il1α and bFGF would render cells unresponsive to DNA-damaging agents. A further prediction is that there would be not difference between, for example, bFGF-dependent and UV-dependent modification of a transcription factor. Such experiments are in progress.

The pathway of signal transduction is bound to yield further surprises. One fact is, however, clear: regulatory proteins must be the targets of the signal transduction mechanism(s) and these targets must be activated (or inactivated) by posttranslational modification.

6 The Regulatory Proteins Targeted by DNA Damage-Induced Signal Transduction

Most of the eukaryotic regulation of genes occurs at the promoter level. Therefore, as expected, most of the genes responsive to DNA damage and for which the molecular analysis has been completed, are regulated by altered promoter activity (Table 1). The frequency of transcriptional initiation is determined by a whole range of features: promoter accessibility (chromatin structure), sequence of the RNA polymerase and accessory protein binding sites, and transcription factors. For those genes that are activated in the absence of protein synthesis, the critical regulatory proteins are the direct endpoints of the damage-induced signal transduction. Cycloheximide-resistant induction would classify as primary response. If members of the primary response genes controlled other genes, their regulation would be inhibited by inhibitors of protein synthesis. There may be exceptions to this rule in that inhibitors of protein synthesis prevent the resynthesis of short-lived proteins. If these were repressors or positive cofactors of transcription, the classification as primary response would be incorrect.

DNA damage may alter chromatin structure and DNA methylation. One could imagine this to occur in the direct neighborhood of the damage or as a result of a *trans*-acting mechanism. Severel reports have claimed such changes resulting in enhanced promoter activity (Lieberman et al. 1983; Barr et al. 1986; Valerie and Rosenberg 1990). The molecular mechanism is not clear.

Table 1. DNA damage-induced genes

Gene	Cell/tissue	Agent	Product examined	Promoter element and transcription factors involved	Remarks	References
Oncogenes						
c-*fos*	HeLa, human fibroblasts, 3T3, F9	UVC (2 – 30 J/m^2)	RNA, protein	−300/−320 +18/+38 Serum response factor and associated proteins	RNA accumulation is cycloheximide resistant	Angel et al. 1985; Büscher et al. 1988
	CHO	MMS (200 µg/ml) MNNG (30 µ*M*) heat shock 4-NQO (1 µg/ml) H_2O_2 (400 µ*M*)	RNA			Hollander and Fornace 1989 Shibanuma et al. 1990
	Colon carcinoma cells	Bifunctional alkylating agents	RNA			Futscher and Erickson 1990
	HL60	X-rays (5 – 50 Gy)	RNA		Not inducible in many other cells	Sherman et al. 1990; Hallahan et al. 1991; Herskind et al. 1991
	Murine epidermal cells (JB6 clone 30)	ROI	RNA		Less inducible in clone 41	Crawford et al. 1988
	HeLa, CHO	Heat shock	RNA, protein		May be due to inhibition of protein synthesis	Andrews et al. 1987; Hollander and Fornace 1989
	Hairless mouse epidermis	UVA/B	RNA			Brunet and Giacomoni 1990
	Syrian hamster embryo cells	X-rays (1 Gy)	RNA		No induction with Janus fission spectrum neutrons	Woloschak and Chang-Liu 1990
	Murine gut tissue	Janus fission spectrum neutrons (0.5 Gy)	RNA			Munson and Woloschak 1990

Table 1 (continued)

Gene	Cell/tissue	Agent	Product examined	Promoter element and transcription factors involved	Remarks	References
c-*jun*	HeLa, 3T3, F9 human fibroblasts	UVC (2 – 30 J/m^2)	RNA, protein	– 71/ – 64 – 190/ – 183 AP-1 like factors	RNA accumulation is cycloheximide resistant	Stein et al. unpublished; Devary et al. 1991; Krämer et al. 1990; Stein et al. 1992
	HL60, U937, human fibroblasts	X-rays (5 – 50 Gy)	RNA			Sherman et al. 1990; Hallahan et al. 1991; Weichselbaum et al. 1991
	HeLa	H_2O_2 (250 μ*M*)	RNA			Devary et al. 1991
jun-B	HL-60	X-rays (20 Gy)	RNA			Sherman et al. 1990
	HeLa	UVC (20 – 40 J/m^2)	RNA			Stein et al. 1992; Devary et al. 1991
c-*myc*	Mouse epidermal cells (JB6 clone 30)	ROI	RNA		RNA accumulation may be due to mRNA stabilization	Crawford et al. 1988
	Rat fibroblasts	UVC (2 J/m^2)	RNA			Ronai et al. 1988
	Lymphoblastoid cells	DMS (0.1 μ*M*) EMS (0.1 μ*M*) Mytomycin C (3 n*M*) X-rays (2 Gy) Bleomycin (1 μg/ml)	Protein			Sullivan and Willis 1989
	Colon carcinoma cells	Bifunctional alkylating agents	RNA			Futscher and Erickson 1990
p53	3T3, human fibroblasts	4-NQO (0.4 μg/ml) UV (5 – 50 J/m^2)	Protein		Stabilization of the protein; stabilization occurs only in cells capable of enhanced viral reactivation	Maltzman and Czyzyk 1984; Abrahams et al. 1984; and: Abstract 4C, 1 in Workshop on DNA Repair, Noordwijkerhout, April 1991

Other genes					
α-interferon	Syrian hamster embryo cells	X-rays (1 Gy)	RNA		Woloschak and Chang-Liu 1990
Interleukin 1	Syrian hamster embryo cells	Neutrons (0.2 Gy)	RNA		Woloschak et al. 1990a
	HeLa	UVC (30 J/m^2)	RNA, protein		Krämer et al. unpublished
bFGF	Aortic endothelial cells	X-rays (4 Gy)	RNA, protein		Haimovitz-Friedman et al. 1991
	HeLa	UVC (30 J/m^2)	RNA, protein		Krämer et al. unpublished
TNFα	Human sarcoma cells	X-rays (5 Gy)	Protein, RNA	Not elevated in human fibroblast cell lines and epithelial tumor cell lines	Hallahan et al. 1989
EGF receptor	Human keratinocytes	UVA ($10^4 - 1.5 \times 10^5$ J/m^2) 8-MOP (0.1 – 2.5 μg/ml)	RNA	May not be due to DNA damage	Yang, X-Y et al. 1988
MHC class I	Human fibroblasts Human keratinocytes	UVC (1 – 20 J/m^2) UVA (1.3×10^5 J/m^2) BPDE (0.1 μg/ml)	Protein		Lambert et al. 1989
Protein kinase C	Human epithelial cells Syrian hamster embryo cells	Solar radiation X-rays (2 Gy)	RNA	Janus fission spectrum neutrons have only a minimal effect	Peak et al. 1991; Woloschack et al. 1990b; Kim et al. 1991
α-tubulin	Syrian hamster embryo cells	γ-rays (2 Gy) neutrons	RNA		Woloschak et al. 1990c
γ-actin	Syrian hamster embryo cells	γ-rays (2 Gy) neutrons	RNA		Woloschak et al. 1990c
β-actin	Syrian hamster embryo cells	γ-rays (2 Gy) neutrons	RNA		Woloschak et al. 1990c
	Hamster cells	MNNG (4 μg/ml)	RNA CAT		Kleinberger et al. 1988

Table 1 (continued)

Gene	Cell/tissue	Agent	Product examined	Promoter element and transcription factors involved	Remarks	References
Ribosomal protein L7a	Human fibroblasts	UVC (1 – 15 J/m^2) 4-NQO (0.1 – 10 μ*M*) DMS (200 m*M*) heat shock	RNA		The gene was found as a recombinant with the *trk* proto-oncogene	Ben-Ishai et al. 1990; Ziemiecki et al. 1990
GAPDH	Human keratinocytes	UVC (35 – 50 J/m^2) 4-NQO (0.5 m*M*)	RNA		RNA is not induced in human or murine fibroblasts, in F9 cells or in HeLa cells	Kartasova et al. 1987 Stein et al. 1992
Collagenase	HeLa cells, human fibroblasts	UVC (2 – 30 J) Mitomycin-C (1 μg/ml) 4-NQO (1.3 μ*M*) X-rays (8 Gy)	RNA, protein	– 72/ – 66 and upstream sites c-Fos/c-Jun (AP-1)	Induction is diminished in the presence of cycloheximide	Mallick et al. 1982; Herrlich et al. 1986; Angel et al. 1986, 1987a, b Stein et al. 1989a
Plasminogen activator (urokinase)	Human fibroblasts Lymphocytes	UVC (1 – 15 J/m^2)	Enzyme activity, RNA			Miskin and Ben-Ishai 1981; Ben-Ishai et al. 1984; Rotem et al. 1987; Sugita et al. 1991
Invariant chain	Myeloma cells Pre-B cells	UVC (17 J/m^2) Mitomycin-C (0.5 μg/ml) X-rays (4.5 Gy)	RNA, protein		No induction of the gene by DNA damaging agents in fibroblasts	Rahmsdorf et al. 1982, 1983, 1986
EGR1	Human epithelial cells Fibroblasts Kidney epithelial cells	X-rays (3 – 20 Gy)	RNA		RNA accumulation is cycloheximide resistant	Hallahan et al. 1991; Weichselbaum et al. 1991
Ornithine decarboxylase	Keratinocytes	UVB ($10^5 – 5 \times 10^5$ J/m^2)	RNA			Rosen et al. 1990
	Syrian hamster embryo cells	Neutrons (0.2 Gy) X-rays (1 Gy)	RNA			Woloschak et al. 1990a
	Tracheal epithelial cells	ROI	Protein, RNA			Marsh and Mossmann 1991

Metallo-thioneins	Fibroblasts	UVC ($2-30\ J/m^2$)	RNA			Lieberman et al. 1983; Angel et al. 1986
	V79	UVC ($14\ J/m^2$)	RNA			Fornace et al. 1988a, b
Heme oxygenase	Various cells	ROI	RNA, protein			Keyse and Tyrrell 1989; Keyse et al. 1990; Applegate et al. 1991; Gomer et al. 1991
O^6-methyl-guanine-DNA-methyl-transferase	Rat hepatoma L929	UVC ($10\ J/m^2$) X-rays (3 Gy) DDP ($5\ \mu M$) Bleomycin ($25\ \mu g/ml$) Heat shock	Protein, RNA			Lefebvre and Laval 1986; Nehls et al. 1991; Fritz et al. 1991
3-methyl-adenine DNA glycosylase	Rat hepatoma	DNA-damaging agents	Protein, RNA			O'Connor and Laval 1990; Habraken et al. 1991: Abstract 4C, 8 in Workshop on DNA Repair, Noord-wijkerhout, April 1991
β-DNA-polymerase	CHO	MNNG ($30\ \mu M$) MMS ($100\ \mu g/ml$) AAAF ($20\ \mu M$) H_2O_2 ($400\ \mu M$)	RNA	$-49/-40$ ATF/CREB	Is not induced by UV-C ($14\ J/m^2$)	Fornace et al. 1989c; Kedar et al. 1991
DNA ligase	Monkey kidney	UV ($9-17\ J/m^2$)	Protein			Mezzina and Nocentini 1982
Ubiquitin	Various hamster and human cell lines	MMS ($100\ \mu g/ml$) DDP ($45\ \mu g/ml$) Heat shock	RNA		X-ray, UV, TPA Do not induce	Fornace et al. 1989a
Collagens	Mice lung	$^{239}PuO_2$	Protein		Long-term effects (months)	McAnulty et al. 1991
	Pig muscle	X-rays (60 Gy)	Protein		Long-term effects (months)	Remy et al. 1991

Table 1 (continued)

Gene	Cell/tissue	Agent	Product examined	Promoter element and transcription factors involved	Remarks	References
P glycoprotein	Tumor cells	Fractionated X-rays (9 Gy fractions)	Protein		No enhancement of RNA	Hill et al. 1990
50K, 56K keratines	Keratinocytes	UVC (35 – 50 J/m^2) 4-NQO (0.5 m*M*)	RNA			Kartasova et al. 1987
DDI-class I	Various human and hamster cells	UVC (14 J/m^2)	RNA		Not induced by MMS	Fornace et al. 1988a; Fornace et al. 1989b
DDI-class II		UVC (14 J/m^2) MMS (100 μ*M*) H_2O_2 (400 μ*M*)	RNA		Not induced by heat shock, TPA, X-rays (except gadd 45), Bleomycin (50 μg/ml), Five clones are induced by serum starvation (gadd 45, gadd 153, A34, A33, A7)	Papathanasiou et al. 1991 Fornace et al. 1991
*spr*I, *spr*II	Human keratinocytes	UVC (35 – 50 J/m^2) 4-NQO (0.5 m*M*)	RNA			Kartasova et al. 1987; Kartasova and Van de Putte 1988; Gibbs et al. 1990
Long interspersed repetitive DNA	Leukemia cells	UV (broad spectrum) X-rays (3 Gy)	RNA		After X-ray RNA level first decreases (4 h) and later increases (24 h)	Servomaa and Rytömaa 1990
Various proteins	Malignant melanoma	X-ray (3 Gy)	Protein			Boothman et al. 1989, 1990; Hughes and Boothman 1991

Interferon α Inducible transcription	Human cells	UV	RNA		mRNA stabilization	Hilgers et al. 1991
P2, P3	V79	Alpha (4.4 Gy)	Protein			Herrlich et al. 1986
RP-2, RP-8	Thymocytes	X-rays (9 Gy)	RNA		Involved in apptosis?	Owens et al. 1991
DNA-binding proteins	HeLa	UVC (12 J/m^2)	Protein		Does not require de novo protein synthesis	Glazer et al. 1989
	Lymphoblastoid cells	X-rays (2.5 – 20 Gy)				Singh and Lavin 1990
Viruses						
HIV-1	HeLa, fibroblasts	UVC (2 – 30 J/m^2)	RNA	Enhancer NFκB	Poorly activated by X-ray	Valerie et al. 1988; Stein et al. 1989a, b;
		4-NQO (1.3 μ*M*) Mitomycin-C (1 μg/ml)			Enhancement more dramatic in stable transfectants	Valerie and Rosenberg 1990; Sadaie et al. 1990
	Human T cells	ROI				Schreck et al. 1991
	Transgenic mice LTR-Lac Z	UVC (60 J/m^2) UVB (60 J/m^2)	β-galactosidase			Cavard et al. 1990
VL30	Mice	Neutrons (0.5 Gy)	RNA			Panozzo et al. 1991
		X-rays (3 Gy)	RNA			
MoMuSV	3T3	UVC (2 – 10 J/m^2) X-rays (1 Gy)	RNA		Downregulation of PKC inhibitis X-ray, but not UV induction	Lin et al. 1990
SV40	HeLa, hamster cells	UVC (30 J/m^2) MNNG (4 μg/ml)	Protein (CAT)	Enhancer		Hagedorn et al. 1983; Vanetti 1988; Kleinberger et al. 1988

AAAF, *N*-acetoxy-2-acetylaminofluorene; *BPDE*, benzo(a)pyrene-*trans*-7,8-dihydrodiol-9,10-epoxide; *DDP*, *cis*-Pt(II)diamminedichloride; *DMBA*, 7,12-dimethyl-benz(a)anthracene; *DMS*, dimethylsulfate; *EMS*, ethylmethane sulfonate; *ENU*, *N*-ethyl-*N*-nitrosourea; *MMS*, methylmethane sulfonate; *MNNG*,*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; *MNU*, *N*-methyl-*N*-nitrosourea; *8-MOP*, 8-methoxy-psoralen; *4-NQO*, 4-nitroquinoline-*N*-oxide; *ROI*, reactive oxygen intermediates; *TPA*, 12-*O*-tetradecanoylphorbol-13-acetate; *UV*, ultraviolet irradiation – A, B, C; *bFGF*, basic fibroblast growth factor; *TNFα*, tumor necrosis factor; *EGF*, epidermal growth factor; *GAPDH*, glyceraldehydephosphate dehydrogenase; *CAT*, chloramphenicol acetyltransferase; *ATF*, adenovirus transcription factors; *CREB*, cyclic AMP response element-binding factor

Transcription factors as endpoints of DNA damage-dependent signaling have been defined by first delimiting the *cis*-acting sequence elements that are required for damage regulation of a gene, and then investigating what binds to these response elements. Using this approach, severel *cis*-acting DNA damage-response elements and their cognate transcription factors have been found (for review see Krämer et al. 1990). To exert its function a transcription factor must bind DNA, usually by recognizing the *cis*-acting element directly, and carry a functionally active transactivation domain that, in an as yet largely unknown fashion, stimulates the basal transcription factor TFIID and other proteins of the transcriptional initiation complex (for reviews see Maniatis et al. 1987; Guarente 1987; Ptashne 1988; Dynan 1989; Mitchell and Tjian 1989). The transcription factors responding to DNA damage are low in spontaneous activity and become active upon "receiving" the damage-induced signal. The signal may cause an increase of DNA-binding activity of the transcription factor (e.g., of AP-1, Stein et al. 1989a) or the functional exposure of the transactivating ability of a constitutively binding factor (e.g., $p67^{SRF}$, Stein et al. 1989a).

How do we know that a transcription factor is indeed modulated directly by components of the damage-induced signaling cascade? As listed in Table 1, the gene c-*fos* is induced by DNA damage (Angel et al. 1985). Several regions of the promoter contribute to the response. The most important DNA damage response element is located between positions −320 and −300 (nucleotides upstream of the start of transcription, Büscher et al. 1988) and known as the dyad symmetry element (Treisman 1985). This element confers also responsiveness to phorbol esters and to serum growth factors. The factors binding to this element cause a genomic footprint. Such analyses have shown that the factor is constitutively present and that there is no change of occupancy after treatment with phorbol esters and serum (Herrera et al. 1989; König 1991). Presumably this will hold for DNA-damaging agents. Thus, this factor must be subjected to direct modulation.

The product of c-*fos*, Fos protein, is itself the subunit of a transcription factor (Schönthal et al. 1988; Nakabeppu et al. 1988; Halazonetis et al. 1988; Rauscher et al. 1988). Fos in its association with Jun (the trancription factor AP-1) regulates a large number of genes. One of these codes for collagenase (type I). UV inducibility of collagenase has been mapped to the AP-1 binding site (Stein et al. 1989a). Artificial promoter constructs that contain nothing but the AP-1 site plus the TATA box are UV responsive (unpublished). Since AP-1 is quite abundant and induction of RNA could not be inhibited by cycloheximide, this has been considered proof for the direct UV-induced posttranslational activation of AP-1. However, cycloheximide alone can induce such promoters (unpublished) perhaps indicat-

ing the existence of a short-lived enzyme which inactivates AP-1. These experiments illustrate the ambiguity of the classification as primary and secondary that has been introduced above. Still AP-1 is modulated by post-translational modification. Changes in the phosphorylation pattern of AP-1 in response to either UV (Gebel, unpublished), phorbol esters (Boyle et al. 1991) or oncogene overexpression (Binétruy et al. 1991) have been implicated as crucial modification steps. Interestingly these patterns differ between UV and phorbol ester, suggesting that the ultimately modifying enzymes differ between these pathways (Gebel, unpublished). Yet, the relationship of modification with function needs to be proven.

Not all DNA damage-responsive genes are regulated by altered promoter activity. There are no examples as yet for regulated transcriptional elongation or splicing following DNA damage. Induced prolongation of mRNA lifetime has been reported in trypanosomes (Pays et al. 1990), suggesting a determinant of mRNA half life as a target of damage-induced modification. Also in mammalian cells UV seems to stabilize certain RNAs (as, for example, interferon α and Il1 α induced mRNAs: G. Hilgers et al. 1991). Translation (Yamagoe et al. 1991) and protein fate (as suggested for p53, Maltzman and Czyzyk 1984) may also be influenced (Table 1).

As for stress-induced gene expression, the amplification of DNA sequences by overreplication occurs as a result of rapid signal transduction. Time course and cycloheximide resistance suggest amplification to result from a signaling mechanism similar to that described for induced gene expression. In vivo an excess of an origin segment of SV40 can block DNA damage-induced overreplication of SV40. This origin fragment binds a protein complex in vitro when incubated with extracts from cells that had been treated with DNA-damaging agents (Lücke-Huhle et al. 1989). Our current assumption is that a protein component of the SV40 origin binding complex is limiting and activated after DNA damage. A complex formed at the Polyoma viral origin has recently been described (Ronai and Weinstein, 1990).

7 Stress-Induced Signal Transductions Must Be Limited: Signal Extinction at Various Levels

Regulation in response to a signal can only occur if the activated state does not persist. Otherwise there would be no response, but rather cells would be permanently stress resistant. The need for self-limitation of a stimulating mechanism becomes even more obvious in view of the fact that the DNA damage-induced transcription factors Fos and Jun are themselves

transforming proteins and their genes "nuclear" oncogenes. Oncogenes must be kept under stringent control, such that remain nontransformed. Cells need to possess devices that limit and extinguish the signals which activate oncogene products.

One could imagine that the process of stress-induced signaling is counterregulated and extinguished at various levels, and, indeed, several levels of counterregulation have been found. It may be appropriate to go over the negative control in "reverse order" (as compared to our presentation of signaling) starting with the transcription factor after its induced synthesis. The transcription factor AP-1 (Fos/Jun) represents a prime example. Both its activity and synthesis are downmodulated.

Both components of AP-1, the proteins c-Jun and c-Fos, are subject to progressive phosphorylation immediately after synthesis (Curran et al. 1984, Barber and Verma 1987; Müller et al. 1987; Franza et al. 1988; Kovary and Bravo 1991). Although the specific effects of most individual phosphorylations have not been dissected, it has been recognized early that certain phosphorylated forms bind less well to DNA, suggesting reduced activity (Müller et al. 1987; Boyle et al. 1991). In addition to covalent modification, various interactions with other transcription factors (e.g., other members of the Fos/Jun family, members of the steroid hormone receptor family, adenoviral E1A and cellular E1A analogues) modulate AP-1 activity (Jonat et al. 1990; Yang-Yen et al. 1990; Schüle et al. 1990a; Kerr et al. 1990; Offringa et al. 1990). The action at individual AP-1 dependent genes can also be controlled by other transcription factors whose binding sites either overlap or operate from an independent location (Mordacq and Linzer 1989; Schüle et al. 1990b). Thus the balance of abundance of oppositely acting transcription factors determines the outcome. It is not known, however, whether the stress induction coactivates such opposing factors, for example, in a somewhat delayed manner.

Even more elaborate counter-control mechanisms exist for the induced synthesis of Fos and Jun. A precondition for these to work is the brief RNA and protein half-life of Fos and Jun (Kovary and Bravo 1991). The degradation seems constitutive and very fast (i.e., 8 min for c-fos RNA and 30 min for c-Fos protein). The kinetics of damage induction of Fos or Jun already show the strict control of the process. Transcription is maximal after 40 to 100 min. Promoter activity is turned off rapidly. Evidence exists for that the products of both genes, Fos and Jun, themselves negatively interfere with transcription (Schönthal et al. 1988; Sassone-Corsi et al. 1988; Schönthal et al. 1989; König et al. 1989; Park et al., unpublished). Expression of Fos and Jun from a hormone-inducible promoter reduces DNA damage or phorbol ester-dependent transcription from both endogenous promoters, while antisense *fos* and *jun* cause the opposite. Interestingly,

negative autoregulation, at least of *c-fos* expression, operates at the transcription factor binding to the DNA damage-inducible promoter element (p67^{SFR}, König et al. 1989). Fos protein interferes without itself binding to DNA, a novel type of action of a DNA binding transcription factor (König et al. 1989; Lucibello et al. 1989). The occupancy of the promoter element does not change during the inhibitory action as determined by genomic footprints (Herrera et al. 1989; König 1991). Thus p67^{SRF} is presumably blocked in its transactivating property only.

In addition to this end-product inhibition of the promoter, preceding steps of the induction mechanism comprise inbuilt limitations. An interesting relevant facet is represented by an intrinsic property of the signal transduction pathway. Once stimulated, e.g., by DNA damage, it becomes refractory to new stimulation by the same agent for several hours (Büscher et al. 1988). The molecular mechanism of the refractory state is not understood. Refractoriness to stimulation is not caused by the negative promoter autoregulation described in the previous paragraph since other inducing agents, also those that act through p67^{SRF}, can still trigger new bursts of *c-fos* transcription. It has been assumed that a component of the signal transduction pathway is used up, e.g. by proteolytic degradation of protein kinase C (PKC) (Rodriguez-Pena and Rozengurt 1984; Krug et al. 1987). PKC disappears with a half-life of at least 4 h, much too slow to account for the state of refractoriness observed here. Obviously the consumption concerns parts of the pathway prior to its merging because phorbol ester-pretreated cells can fully respond to UV and vice versa (Büscher et al. 1988). The property of refractoriness may be of use for the dissection of pathways: agents that cause cross-refractory states will stimulate the same branch of the signaling path.

8 Oncogene-Driven Mutagenesis as One of the Long-Term Consequences of Stress-Induced Gene Expression

Both stress-induced overreplication of genes and stress-induced gene expression have long-term consequences which may alter the cellular phenotype and the genetic constitution of cells. Since only a fraction of the stress-induced program of gene expression is known to date, the full complement of the phenotypic properties cannot be foreseen. We can, however, try to extrapolate from the genes identified (Table 1) and from the spectrum of transcription factors activated.

The activation of members of the NFκB and Fos-Jun families addresses possibly an extensive program of dependent genes. While NFκB regulates

many of the genes involved in the control of the immune response and in inflammatory processes, the Fos-Jun family is linked to genes involved in proliferation and transformation, but also to genes of differentiation pathways. The best-studied Fos-Jun-dependent genes are the metalloproteases collagenase and stromelysin. The activity of these two enzymes is critical in the relationship of cells with the extracellular matrix. Metalloproteases are usually synthesized as proenzymes in balance with activating and inhibiting proteins. By blocking the synthesis of the major inhibitor TIMP (tissue inhibitor of metalloprotease by the antisense technique), the importance of fine regulation of this balance was deduced. Upon removel of TIMP, the cells adopt a partially transformed phenotype, namely, anchorage-independent growth and soft agar colony formation (Khokha et al. 1989). Another feature of metalloprotease activity has been revealed by overexpressing collagenase type I or stromelysin in rat2 cells that had been transformed by the activated ras gene. The tumors in the animal grew larger and were more vascularized as compared to the appopriate controls (Giles, Ponta et al., unpublished). Also the overexpression of UV-inducible plasminogen activator in tumor cells led to a change in the growth behavior of the tumors and to spreading ability (Mignatti et al. 1986; Ossowski 1988). Plasminogen activator may act by activating metalloproteases. Vice versa, inhibition of metalloprotease expression by E1A led to an inhibition of the metastatic phenotype (Pozzati et al. 1988; Frisch et al. 1990).

NFκB controls the expression of the HIV-1 virus, the etiological agent of AIDS (Nabel and Baltimore 1987; Dinter et al. 1987). Various DNA-damaging agents (UV irradiation, 4-nitroquinoline oxide, reactive oxygen intermediates, ROI) activate the transcription factor NFκB and lead to enhanced expression of HIV-1 (Table 1). Since in the organism HIV-1 is expressed predominantly in CD4-positive T cells and macrophages, which are not reached directly by UV irradiation, we asked whether UV irradiation in humans may be at all relevant for the development of the disease and examined the culture medium of UV-treated cells for its capability to induce the expression of HIV-1 in epithelial cells and in T cells. These experiments have shown that UV-treated cells indeed secreted an activity which induces HIV-1 transcription (Stein et al. 1989b). Probably the UV-induced secretion of Il1α or bFGF from UV-treated cells accounts for this activity. These findings led us to assume that cells inside the organism harboring the HIV-1 virus can be reached via UV irradiation of the organism through diffusible proteins and that one of the long-lasting consequences of the mammalian stress response may be shortening of latency of virus-dependent diseases.

Perhaps to our surprise (because in contrast to bacteria and yeast), repair genes so far identified do not appear to be DNA-damage inducible.

There are some rather marginal exceptions: metallothioneins (Angel et al. 1986; Fornace et al. 1988a, b) and O^6-methylguanine-DNA methyltransferase (Fritz et al. 1991). These genes are moderately induced by various DNA-damaging agents. Metallothionein II A and O^6-methylguanine-DNA methyltransferase, when overexpressed, reduce alkylation toxicity (Kaina et al. 1990, 1991). Also the inducibility of DNA-polymerase-β (Fornace et al. 1989c) and DNA ligase (Mezzina and Nocentini 1978) may be part of a repair reaction. We expect that other repair genes once they have been isolated will be detected as DNA damage inducible. An indication is given by the finding that the injection of crude extracts from UV-treated (but not from untreated) cells induces unscheduled DNA synthesis (Hoeijmakers, personal communication).

Can DNA repair occur efficiently if cells are pushed through the cell cycle at the same time? This would seem to be the effect of elevated c-fos, c-jun, c-myc and of other immediate response genes. p53, however, could have the opposite effect: delay of cell cycle progression (Diller et al. 1990). A delay in G1 would be beneficial for DNA repair.

For humans the most threatening consequence of the action of most DNA-damaging agents is the development and spread of cancer cells. Without going into the various types of circumstantial evidence that link genotoxic agents with cancer, it is quite obvious that the genetic changes found in cancer cells could well be explained as being induced by DNA-damaging agents: point mutations in dominant oncogenes, promoter mutations of dominant oncogenes, oncogene amplifications, various types of chromosomal aberrations that involve oncogenes or tumor suppressor genes (recent reviews by Bishop 1991; Marshall 1991). The question here, for the topic of this review, is whether the genetic changes are direct consequences of DNA damage or whether the proteins induced in the stress response, are involved in their development; that is, whether mutation induction depends on stress-induced protein synthesis (as has been found in bacteria).

The point mutations in cellular oncogenes provide the most convincing evidence for DNA damage specificity and thus presumably direct involvement of a specific DNA lesion as a precursor of the mutation. Specific point mutations have been found in transformed cells after treatment with 7,12-dimethyl-benz(a)anthracene, X-ray, UV, *N*-ethyl-*N*-nitrosourea and *N*-methyl-*N*-nitrosourea (reviewed by Barbacid 1987). These mutations match the type expected from the mechanism of mutagenesis by these agents that had been derived from bacterial studies, and the lesions known to be caused in mammalian cells (J.-L. Yang et al. 1988). From bacteria, however, we also know that there is no mutation in the absence of an SOS response (reviewed by Walker 1985). We may ask whether the agent speci-

ficity of mutations can be reconciled with the possible existence of an error-prone repair or mutator function.

Hints that induced mutator mechanisms may exist in mammalian cells have been reported off and on:

1. Enhanced mutagenesis and reactivation of viruses in cells that had been pretreated with a DNA-damaging agent (DasGupta and Summers 1978; Cornelis et al. 1982; Defais et al. 1983; Abrahams et al. 1984; Herrlich et al. 1984).
2. Pulse mutagens have been found to induce a state of mutation proneness for many cell generations (Maher et al. 1988; Little et al. 1990; Little and Krolewski 1991). The basis for this cellular state has, however, not been discovered. It may be due to the stable induction of a mutator protein, to the persistence of lesions somewhere in the genome, or to the continued replication and reintegration of endogenous proviruses. Since a search for the mutants produced has been unsuccessful or the mutants have not been analyzed, neither possibility can be ruled out.
3. An intriguing experiment has been the obviously nontargeted introduction of mutations by conditioned medium from UV-treated cells (Maher et al. 1988). In another report, conditioned medium enhanced the generation of targeted mutations (Dixon et al. 1988). The magnitude of the enhancement was not great, 2- to 4-fold in both studies. It is not yet known which component of the conditioned medium from UV-treated cells is involved in the induction of mutations. Although it is possible that secreted growth factors lead to the induction of mutator proteins, there are alternative interpretations. Growth factors such as interleukin 1α and TNF have been described to also induce the generation of ROIs in various cells including fibroblasts (Meier et al. 1989, 1990). These ROIs may directly cause DNA damage.

The work with transgenic or chimeric mice (Hanahan 1988; Wagner 1990; Bailleul et al. 1990; Aguzzi et al. 1991) has shown that, in addition to an activated dominant oncogene introduced into mice, probably more than one additional genetic step needs to occur to produce a cancer cell. As a result not each cell transcribing the oncogene is transformed but rather a clonal minority. These additional steps (mutations) would be the result of endogenous processes in the absence of a DNA-damaging agent (although unavoidable DNA damage cannot be ruled out). Transgenic experiments thus distinguish the genes introduced from the other steps leading to additional mutations, and suggest that activated oncogenes may induce a state of error proneness in mammalian cells.

Since DNA-damaging agents induce the synthesis of various oncoproteins (c-*Fos*, c-*Jun*, c-*Myc*), we decided to attempt to separate the synthesis

of these proteins from DNA damage and ask whether the overexpression of these proteins in cells (without DNA damage) causes mutations. In a first series of experiments we analyzed the effect of overexpression of AP-1. This was based on the assumption that DNA damage-induced AP-1 would account for a large part of the stress response, perhaps including mutator functions. AP-1 activation can be achieved by the experimental overexpression of either Fos or Jun or both. In our preliminary study we overexpressed Fos under the control of the dexamethasone-inducible mouse mammary tumor virus long terminal repeat (MMTV LTR), thus generating active transctiption factors that contain Fos as subunit, including AP-1. In the cells used (NIH3T3) there is a significant spontaneous level of Jun but no detectable Fos. In response to dexamethasone, Fos is synthesized and the cells do accumulate, obviously in response to the pulse expression of Fos, for a few hours, mutations at the Na^+/K^+ ATPase and the hypoxanthine-guanine-phosphoribosyltransferase (HGPRT) loci. The enhancement was quite considerable, from 3- to 40-fold (van den Berg et al. 1991).

In a similar approach we assayed for the generation of cytogenetically visible chromosomal aberrations after pulse expression of Fos. Fos induced by either dexamethasone or cadmium (from appropriate promoters), led to elevated levels of aberrations, mainly gaps, in the next metaphase (van den Berg et al. 1991). These data suggest that the Fos-dependent portion of the stress response includes proteins that act directly, or possibly through an extracellular loop, on DNA and cause chromosome and gene mutations.

If mutagenesis dependend on such a function, the generation of DNA damage-induced mutations should not work in cells that had been deprived of Fos protein. In experiments with antisense fos, UV-induced chromosomal aberrations were indeed reduced (van den Berg et al. 1991).

These experiments led us to assume that the stress response participates in the generation of mutations. While these data suggest at the same time that normal growth and stimulation by growth factors and inflammatory mediators may be mutagenic, a note of caution should be mentioned: it is still possible that the effects observed are produced by appropriate DNA damage directly if we postulate that Fos causes bursts of reactive oxygen intermediates and radicals that attack DNA. Further studies are required to unravel the molecular mechanisms of oncogene-induced mutagenesis.

Mutagenesis studies have, to our knowledge, so far not revealed a threshold dose below which there is no effect. How could this be explained in view of a putative involvement of the stress response in mutagenesis and repair? A possibility is that the stress response is also elicited in a linear dose dependence. It may also be possible that a minimum signal flow is needed to generate the activation above the spontaneous level of active fac-

tor (AP-1 or else). In this case a threshold for the induction of mutations would be generated. From the experiments with nondamaged or with damaged viral DNA that had been inserted into pretreated cells (Seidman et al. 1987) we know that targeted mutations are still the vast majority. Since targeted mutations may not depend in all cases on the presence of the mutator functions, the generation of targets may define the dose response curves and conceal the putative threshold.

Conclusions

In mammalian cells, as in bacteria, DNA-damaging agents induce overreplication and a new program of gene expression. An increasing number of induced proteins have been identified; still, most physiologic functions of stress-induced proteins are unknown. Elements of the regulatory mechanism have, however, been unraveled. DNA damage appears to be the major primary event. Transcription factors are posttranslationally modified. Some components of signal transfer have been identified: a growth factor loop delivers signals to the irradiated cell and to neighboring cells. With respect to long-lasting consequences of the DNA-damage response, an important observation concerns the DNA damage-induced increase in abundance of proteins that participate in the generation of mutations. These proteins can be upregulated by oncogenes in the absence of DNA damage. This process may be relevant for the mechanism of tumor promotion and tumor progression.

Acknowledgements. We thank Udo Mallick, Marina Schorpp, Peter Angel, Marita Büscher, Bernd Stein, Marcus Krämer, Bernd Kaina, Susanne van den Berg, Sabine Mai and Christine Lücke for collaborating with us on the DNA damage-induced response.

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Rev. Physiol. Biochem. Pharmacol., Vol. 119
© Springer-Verlag 1992

Transcription Factor Encoding Oncogenes

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1 Introduction: Nuclear Oncoproteins and the Control of Transcription

One of the most intriguing discoveries of the past 4 years was the identification of nuclear oncogene products as transcription factors or subunits of transcription regulatory complexes. Although it had been speculated for some time that nuclear oncoproteins may figure in the regulation of gene expression, there was no definitive proof of this hypothesis prior to the identification of Jun and Fos as constituents of the transcription factor AP-1 (see below for references). This connection between two nuclear oncogene products, originally identified as retroviral transforming proteins, and a known transcription regulatory protein complex can be considered a paradigm of transcription factor-encoding oncogenes. In fact, there is very good evidence to believe that most, if not all, "nuclear proto-oncogenes" encode transcription regulatory factors. In some cases such associations are proven, or seem very likely, and these are the genes that will be discussed in detail in this review: (1) the *fos* and *jun* genes mentioned above, (2) the *rel* gene which encodes a protein resembling the two subunits of the transcription factor NF-κB and the Drosophila morphogenetic protein dorsal, (3) the *myc* gene which was one of the first oncogenes discovered, but whose function in transcriptional regulation was not revealed until very recently, (4) the *erbA* gene which codes for a thyroid hormone receptor whose oncogenic activation involves its conversion into a transcriptional repressor, and finally (5) the *myb* and *ets* genes whose encoded proteins now also turn out to partake in transcriptional regulation. There are several other "nuclear proto-oncogenes" which may well be engaged in some aspect of transcriptional control as well, such as ski (Colmenares and Stavnezer 1989) or *evi*-1 (Morishita et al. 1988), but there is no direct evidence for this at the moment. These genes have therefore been excluded from this review. It should also be mentioned that the products of several anti-oncogenes, also referred to as tumor suppressor genes, appear to be involved in transcriptional regulation. One of these is the retinoblastoma gene product Rb-1, which forms complexes with regulatory viral proteins, such as the adenovirus E1a protein (Whyte et al. 1988), and which has been shown to repress the c-*fos* promoter (see below). Another example is a Wilms' tumor-associated gene product (Gessler et al. 1990), a sequence-specific DNA binding protein whose zinc finger domain has been reported to be impaired in tumor cells (Rauscher et al. 1990). It thus seems that the regulation of transcription involves the interplay of proteins which not only have antagonistic transregulatory functions but often are endowed also with either oncogenic or anti-oncogenic properties.

Transcription factors can be classified according to the structure of their DNA-binding site and the mechanisms by which they recognize specific

DNA elements. The DNA-binding domains usually show a high degree of conservation among different proteins of the same family, so that a given DNA element is frequently recognized by several different transcription factors (for a review see Mitchell and Tjian 1989). According to our current knowledge, all proto-oncogenes encoding transcriptional regulators are members of multigene families. This means that the identification of a given oncogene product as a transcription factor almost naturally led to the discovery of other genes encoding related proteins which often also turned out to be potential oncogenes. The following review of transcription factor-encoding oncogenes is therefore also a discussion of the respective families, although in most cases one family member is the most prominent one and thus discussed in greater detail.

2 *jun:* A Multigene Family of Transcriptional Regulators of AP-1 Dependent Transcription

The transcription factor AP-1 was originally identified as an activity which binds to the SV40 early promoter region and which is induced by tumor-promoting phorbol esters (Angel et al. 1987; Lee et al. 1987). AP-1 consist of a variety of different proteins forming heterodimeric Fos-Jun or homodimeric Jun-Jun complexes, one of the major constituents being c-Jun (Bohmann et al. 1987; Angel et al. 1988). c-Jun is identical with the major moiety of the previously identified Fos-associated protein p39 (Curran and Teich 1982; Curran et al. 1985; Rauscher et al. 1988a; Chiu et al. 1988; Sassone-Corsi et al. 1988a; for a review see Curran and Franza 1988). AP-1 binds to a palindromic recognition sequence, TGACTCA, termed AP-1 site or TPA responsive element (TRE). Although Jun homodimers show specific binding to this element, the affinity of Fos-Jun heterodimers is more than 30fold higher (Nakabeppu et al. 1988; Halazonetis et al. 1988; Rauscher et al. 1988b). The mechanism of Fos/Jun/TRE complex formation is similar to the interaction of other bZip (basic region-zipper) proteins with their cognate DNA element. It involves the formation of protein dimers via the parallel association of the leucine zippers, which in turn leads to the juxtaposition of the basic regions of the two interacting proteins, thus forming a bipartite DNA binding site (Landschulz et al. 1988; Turner and Tjian 1989; Gentz et al. 1989). This issue will be addressed in more detail below (see section on Fos). Jun also forms complexes with members of the cyclic AMP responsive element binding protein (CREB) family, and such heterodimers bind preferentially to the

cyclic AMP response element (CRE), TGACGTCA (Benbrook and Jones 1990; MacGregor et al. 1990; Ivashkiv et al. 1990). Recently, regulatory interactions between Jun, Fos and the receptors for vitamin A and vitamin D (Owen et al. 1990; Schüle et al. 1990a, b) as well as with the glucocorticoid receptor (Diamond et al. 1990; Jonat et al. 1990; Lucibello et al. 1990; Schüle et al. 1990a, b; Yang-Yen et al. 1990) have been reported. Although the molecular mechanisms underlying these interactions are not clear, there is sufficient evidence to suggest that they may be fundamentally different.

One of these recently discovered regulatory mechanisms involving steroid hormone receptors is the mutual transrepression of Fos or Jun and the glucocorticoid receptor (GR) (Jonat et al. 1990; Lucibello et al. 1990; Schüle et al. 1990a; Yang-Yen et al. 1990). The mechanism of Fos-GR repression remains elusive, since formation of physical complexes between the two proteins could not be detected (Diamond et al. 1990). In contrast, interaction between Jun and the GR was observed (Diamond et al. 1990), and Jun protein was shown to disrupt the binding of the GR to the GR element (GRE; Schüle et al. 1990a).

A different regulatory mechanism seems to exist in the case of the proliferin gene promoter, where both a GRE and a TRE are contained within a 25-bp element (Diamond et al. 1990). The effect of glucocorticoids on this element depends strongly on the presence of Fos and Jun proteins. In the absence of Fos and Jun, GR cannot bind and shows no effect. In the presence of Jun, cooperative binding appears to occur with the result of strong transcriptional activation. The addition of large amounts of Fos blocks transcription, suggesting that Fos/Jun/GR complexes are inactive.

In the human osteocalcin gene, a TRE is contained within a DNA element mediating the response to vitamin D and retinoic acid (Schüle et al. 1990b). Transcriptional activation via this element by these ligands is blocked by the coexpression of Fos and Jun, suggesting that functionally antagonistic transcription factors compete for the same element.

While the DNA contact site and dimerization interface of Jun and other bZIP proteins are structurally and functionally well defined, the precise position and nature of the transcriptional activator domains is less clear. Two regions in Jun have been identified as playing a role in transactivation, i.e. a domain at the amino terminus (A1 domain) and an acidic region (A2 domain) adjacent to the basic DNA contact site (Angel et al. 1989; Bohmann and Tjian 1989; Bos et al. 1990; Ransone et al. 1990). Transactivation by Jun is regulated by an inhibitor which binds to the amino terminally located δ region and appears to act predominantly on the A1 activator region (Baichwal and Tjian 1990). In addition, the activity of Jun seems to be controlled by phosphorylation which is stimulated by treat-

ment with growth factors (Turner et al. 1991). Phosphorylation at three sites directly adjacent to the DNA contact site in c-Jun has been suggested to keep the protein in a latent inactive form, since the activation of protein kinase C leads to the dephosphorylation of these residues associated with a 10fold increase in transactivation (Boyle et al. 1991). It has previously been suggested that phosphorylation of the c-Jun/c-Fos complex results in decreased DNA binding (Müller et al. 1987). In addition, O-glycosylation of Jun protein has been described to affect its function as a transcriptional activator (Jackson and Tjian 1988), and DNA binding may be regulated by a redox mechanism (Abate et al. 1990a, b). Finally, a negative regulator of AP-1 interaction with its cognate DNA element has been discovered and termed IP-1 (inhibitor protein-1; Auwerx and Sassone-Corsi 1991), but the precise mechanisms underlying the inhibition of DNA binding remain to be investigated.

As mentioned above, Jun constitutes a multigene family whose members are endowed with different transcriptional regulatory properties and whose expression is differentially regulated. While c-*jun* and *jun*B are rapidly and transiently induced by growth factors, *Jun*D is expressed at relatively high levels in growth factor deprived cells, and its expression increases only slightly after stimulation. Treatment of cells with different inducers of DNA synthesis results in patterns of expression that are characteristic of the three *jun* family members (Ryseek et al. 1988; Chiu et al. 1989). Likewise, distinct patterns of expression are observed during embryonal development for each of the *jun* genes (Ryder et al. 1988; Hirai et al. 1989; Wilkinson et al. 1989). The members of the *jun* family differ markedly in their ability to activate AP-1-dependent transcription: while c-Jun is an efficient activator, JunD is less active, and JunB fails to transactivate and even blocks activation and transformation by c-Jun (Schütte et al. 1989; Chiu et al. 1989). When co-expressed with exogenous Fos, however, JunD forms heterodimeric complexes which transactivate as efficiently as Fos/Jun. These observations point to a delicate network of regulatory mechanisms governing AP-1-dependent transcription through the selection of specific combinations of Fos and Jun family members.

Viral and cellular Jun proteins differ by the presence of gag sequences in v-Jun, the deletion of 27 amino acids and three single amino acid substitutions (Nishimura and Vogt 1988). To activate the transforming potential of c-Jun, the 27 amino acid deletion in the N-terminal half of the protein suffices. This deletion affects the δ region (see above) and is therefore thought to eliminate the interaction with a cellular repressor (Bohmann and Tjian 1989; Baichwal and Tjian 1990). This observation would also suggest that the transactivation of AP-1-dependent transcription plays an important role in Jun-induced transformation.

3 *fos:* A Transforming Gene Encoding a Protein with Multiple Regulatory Properties

According to the prevailing hypothesis, the c-Fos protein acts at a central position in intracellular signal transduction by initiating the regulation of gene expression in response to external signals and is therefore considered a "master switch" (for reviews see Müller 1986; Verma 1987; Herrlich and Ponta 1989; Morgan and Curran 1989). It has been well documented that the c-*fos* gene is induced by a vast number of different molecules which elicit extracellular signals acting through a variety of different transduction cascades. These include the breakdown of phospholipids, the activation of protein kinase C, the intracellular release of Ca^{2+} ions, the influx of monovalent cations, the synthesis of cyclic nucleoside monophosphates and the activation of steroid hormone receptors by ligand binding. It has been suggested that the function of c-Fos is the conversion of short-term signals into long-term genomic responses (Curran and Franza 1988). Due to its central position in intracellular signal transduction, c-Fos also seems to play a crucial role in transformation by other oncogenes. This is not only suggested by the induction of the c-fos gene by a variety of other oncogene products (Schönthal et al. 1988; Gutman and Wasylyk 1991), but also by the resistance of flat revertants of Fos-transformed cells to the transforming action of other oncogenes like *ras* (Zarbl et al. 1987), and by the partial reversion of *ras*-induced transformation by antisense *fos* RNA (Ledwith et al. 1990). In this context, the repression of the c-*fos* promoter by the tumor suppressor gene product Rb-1 is worth mentioning (Robbins et al. 1990).

Research of the past 2 years has provided overwhelming evidence that Fos plays a pivotal role in the regulation of transcription. Following the observation that c-Fos is present in a transcription complex interacting with the promoter of the adipocyte aP2 gene (Distel et al. 1987), it was shown that the target site in the aP2 promoter is a DNA recognition sequence for the transcription factor AP-1, referred to as TPA-esponsive element (TRE) (for a review see Curran and Franza 1988). It is now clear that both c-Fos and the product of another proto-oncogene, c-*jun*, are major components of this transcription factor (Bohmann et al. 1987; see above). The two proteins form heterodimeric complexes which bind with high affinity to the TRE (Nakabeppu et al. 1988; Halazonetis et al. 1988; Kouzarides and Ziff 1988; Rauscher et al. 1988a). Interaction of the two proteins is brought about by hydrophobic interactions between amino acid side chains in the leucine repeates present in both proteins, the leucine zipper (Landschulz et al. 1988; Kouzarides and Ziff 1988; O'Shea et al. 1989; Schuermann et al. 1989; Gentz et al. 1989; Turner and Tjian 1989). The

specificity of interaction is determined by nonleucine amino acids in the zipper, which in the three-dimensional structure of the helix are located adjacent to the leucines (Smeal et al. 1989; Schuermann et al. 1991). The formation of heterodimeric Fos/Jun complexes is a prerequisite for the stimulation of AP-1-dependent transcription by Fos (Schönthal et al. 1988; Lucibello et al. 1988; Sassone-Corsi et al. 1988a; Chiu et al. 1988; Schuermann et al. 1989). In contrast to the high affinity TRE-binding of the Fos/Jun heterodimer, Jun homodimers show considerably lower affinity, and Fos protein is unable to bind to the TRE because of its failure to form homodimers (Nakabeppu et al. 1988; Halazonetis et al. 1988; Rauscher et al. 1988b). The latter conclusion is supported by the observation that a Fos protein containing the leucine repeat from Jun can form homodimers that bind specifically to the TRE (Neuberg et al. 1989a).

The DNA-binding site in Fos was identified as a stretch of predominantly basic amino acids lying immediately adjacent to the leucine repeat. Mutations in this region impair the DNA-binding properties of Fos without affecting the formation of heterodimers with Jun (Kouzarides and Ziff 1988; Neuberg et al. 1989b; Gentz et al. 1989; Turner and Tjian 1989). It is believed that the DNA-binding site of the Fos/Jun complex is bipartite, with Fos and Jun contributing to this domain (Risse et al. 1989; Abate et al. 1990a). This view is supported by the partial sequence homology in the basic regions of Fos and Jun, especially the identical spacing to the leucine repeat and the nearly symmetrical nature of the TRE. The "scissors grip" model addresses the interaction of bZip protein dimers with dyad-symmetric DNA recognition sequences (Vinson et al. 1989). This model predicts that the helices of the two interacting proteins disengage beyond the leucine zipper due to the repulsion of the positively charged surfaces in the adjacent basic DNA-binding domains. The α-helices of the two interacting proteins are suggested to bifurcate and to form Y-like-shaped structures. The bifurcation point is thought to meet the DNA at the center of the dyad-symmetric site, thus enabling the "two arms of the Y" to interact with the two half sites of the DNA recognition sequence by tracking in opposite directions along the major groove of the DNA. Recently, experimental evidence supporting the "scissors grip" model was obtained, based on the dimerization and DNA-binding characteristics of synthetic or purified leucine zipper containing protein fragments (Talanian et al. 1990; Shuman et al. 1990; O'Neill et al. 1990).

In addition to its role in the transactivation of AP-1-dependent transcription, Fos protein has been shown to possess transrepression properties. The mechanisms of Fos-mediated transrepression are fundamentally different from the transactivating properties described above. The c-*fos* promoter is subject to autorepression, however, the target site is not a TRE

but the serum response element (SRE; Sassone-Corsi et al. 1988b; König et al. 1989; Lucibello et al. 1989), the major mediator of c-*fos* induction by growth factors (Treisman 1986). While the involvement of the leucine zipper is controversial at the moment (which may be due to the analysis of two different SRE-containing promoters in the two studies, i.e., c-*fos* and *egr*-1), it is clear that repression of the SRE does not require a functional DNA-binding site (Lucibello et al. 1989; Gius et al. 1990). This suggests that transrepression may involve the titration and inactivation of proteins required for induction of the c-*fos* promoter. The direct target, however, does not seem to be the serum response factor (SRF) itself; the precise mechanism of c-fos autorepression is not understood at present. Interestingly, the transrepressing function of the c-Fos protein is regulated by the phosphorylation of C-terminal serine residues (Ofir et al. 1990). Another recently discovered transrepression mechanism is the mutual transrepression of Fos and the GR (Lucibello et al. 1990; Jonat et al. 1990). For this type of repression both the DNA-binding site and the leucine repeat are dispensible; however, a domain located in the N-terminal half of Fos plays a crucial role in GR repression (Lucibello et al. 1990).

The *fos* oncogene was detected originally in two mouse osteosarcoma viruses, in the Finkel-Biskis-Jinkins mouse osteosarcoma virus (FBJ-MSV), and in the Finkel-Biskis-Reilly mouse osteosarcoma virus (FBR-MSV), suggesting that *fos* is osteochondrotropic with regard to the induction of transformation (Finkel et al. 1966, 1975). This conclusion is in agreement with the apparent involvement of the c-*fos* gene in bone development. While physiologically the c-*fos* gene is expressed at high levels specifically in the cartilaginous growth zone of fetal bone (Dony and Gruss 1987), its deregulated expression in transgenic mice interferes with normal bone development by perturbing the bone remodeling process (Rüther et al. 1987). Despite our increasing knowledge of the molecular properties of the c-Fos protein, we are far from understanding the mechanism by which Fos induces transformation. In mesenchymal cells, which represent the targets for transformation by Fos, the endogenous c-*fos* gene is subject to stringent control mechanisms and is only transiently induced by growth factor signals (Müller 1986). On the other hand, the normal c-Fos protein possesses transforming properties: structural alterations in the regulatory upstream elements and the RNA destabilizing 3′ noncoding region suffice to activate the transforming potential of c-*fos* (Miller et al. 1984; Meijlink et al. 1985). Additional alterations in the coding region, as they occur in the viral *fos* genes, have an enhancing effect on the transforming potential, but are not a prerequisite (Jenuwein and Müller 1987). It therefore appears that the induction of transformation by Fos protein is a consequence of its deregulated expression. The transformation-relevant cellular targets of Fos

are, however, unknown. Likewise, it is not clear which of the known molecular functions of Fos are relevant for the induction of transformation. It seems to be clear that the transregulatory properties of c-Fos as they occur in c-*fos* autoregulation are not relevant for transformation, since v-Fos oncoprotein is unable to repress (Lucibello et al. 1989). On the other hand, both an intact leucine zipper and a functional DNA-binding site are required for the induction of transformation (Schuermann et al. 1989; Neuberg et al. 1991), suggesting that the formation of heterodimeric complexes is crucial. There is, however, no clear correlation between the transformation and transactivating properties of mutated or truncated Fos proteins, in that transactivation is not sufficient for the induction of transformation (Lucibello et al. 1991). These observations suggest that transactivation of TRE-driven transcription is not, or not the only mechanism, involved in transformation. It is possible that transactivation plays a crucial role, but it may affect different target genes. This is conceivable in view of the fact that another DNA element binding Fos protein complexes, the transforming growth factor-β inhibitory element (TIE), has already been identified (Kerr et al. 1990). Alternatively, one could envisage an involvement of multiple molecular functions in transformation, one of which may be the activation of AP-1-dependent transcription. Another mechanism that might play a role is the interference of Fos with steroid hormone receptors, which, at least in the case of the glucocorticoid receptor, involves N-terminal sequences in Fos protein, which are also required for transformation (Lucibello et al. 1990). A clarification of these questions has to await the identification of genes that are regulated by Fos and are instrumental in the induction of transformation. It should, however, also be noted that the situation may be even more complex in view of recent observations suggesting a function for Fos and Jun in DNA replication (Wasylyk et al. 1990a; Murakami et al. 1991).

4 *fos*B: A *fos* Family Member Encoding Antagonistic Transregulatory Proteins

Of the known *fos*-related genes *fra*-1 (Cohen and Curran 1988), *fra*-2 (Matsui et al. 1990; Nishing et al. 1990) and *fos*B (Zerial et al. 1989), the latter seems to be the most interesting one at the moment since it encodes two antagonistic proteins as a consequence of alternative splicing. Induction of the *fos*B gene is part of the "immediate early" response of cells after stimulation with growth factors (Zerial et al. 1989). In addition to the known long form (*fos*B-L), encoding a protein of 338 amino acids

(Fos B-L), a second *fos* B mRNA (*fos* B-S) has recently been identified (Nakabeppu and Nathans 1991; Mumberg et al. 1991). This mRNA is generated by alternative splicing and encodes a product (Fos B-S) lacking the 101 C-terminal amino acids present in the longer Fos B-L protein. This truncation leaves the leucine zipper and the adjacent basic region intact, which is reflected by the ability of both Fos B-L and Fos B-S protein to bind cooperatively with c-Jun to the TRE. The two *fos* B mRNA forms are differentially expressed following serum stimulation, the long form preceding the short form by approximately 30 min (Mumberg et al. 1991). This observation deserves particular attention in view of the fact that the two forms of Fos B differ in their transforming and their transrepressing properties: only Fos B-L is able to repress a c-*fos*-promoter chloramphenicol acetyltransferase (CAT) reporter construct, and Fos B-S can suppress the transrepressing effect of *fos* B-L. Likewise, only the long form efficiently induces transformation and this function is also antagonized by Fos B-S (Mumberg et al. 1991). These functional differences suggest that the transregulatory action of Fos B-L, activated upon serum stimulation, is counteracted by the subsequently expressed Fos B-S. However, Fos B-L and Fos B-S do not differ in all transregulatory properties: transactivation of a 5×TRE-CAT reporter construct in HeLa and NIH3T3 cells was found with both Fos B forms (Mumberg et al. 1991), whereas in F9 embryonal carcinoma cells only the long form of Fos B was able to transactivate a TRE reporter construct in the presence of exogenous c-Jun (Nakabeppu and Nathans 1991). These observations suggest a correlation between *fos* B-induced transformation and transrepression, thus pointing to different mechanisms involved in transformation by *fos* B and c-*fos*/v-*fos*.

It is conceivable that the cell has devised additively acting regulatory mechanisms which keep the potentially oncogenic action of Fos proteins under constraint. It has already been shown that transcriptional activation occurs only at a precise time and only for a few minutes following growth factor stimulation (Greenberg and Ziff 1984; Müller et al. 1984; Kruijer et al. 1984), the mRNA is rapidly degraded with a half-life of about 10 min (Rahmsdorf et al. 1987; Shiu et al. 1988), and Fos proteins turn over with short half-lives of 30–120 min (Curran et al. 1984; Müller et al. 1984). In addition, transrepression by Fos protein has been shown to be dependent on the phosphorylation status of the protein (Ofir et al. 1990). The consecutive expression of an oncogenic transregulator and an anti-oncogenic antagonist derived from the same gene represents an additional mechanism regulating the action of Fos family members.

5 *rel:* An Oncoprotein Resembling the DNA Binding Subunit of Transcription Factor NF-κB and the Drosophila Morphogen Dorsal

The v-*rel* gene is the oncogene of reticuloendotheliosis virus strain T, Rev-T, a replication-defective retrovirus of turkey origin that rapidly induces leukemia in young birds (Theilen et al. 1986). Transformation by Rev-T is specific to avian lymphoid cells, where the 59-kDa v-Rel phosphoprotein is located in the cytoplasm. In fibroblasts, which are refractory to v-Rel transformation, the protein localizes to the nucleus (Gilmore and Temin 1986; Rice et al. 1986; Simek and Rice 1988; Warlo et al. 1987). The c-*rel* gene is a member of a family which comprises at least two other partially homologous genes encoding transcriptional activators, the transcription factor NF-κB and the Drosophila morphogen dorsal. NF-κB is formed by two protein subunits, p50 and p65 (for a review see Gilmore 1990). Both subunits of c-*rel*, p50 and p65, show approximately 60% homology to v-Rel in the region encompassing the DNA binding and dimerization sites (ca. 330 amino acids; Ghosh et al. 1990; Kieran et al. 1990; Nolan et al. 1991). An even greater homology is observed between Rel and dorsal, showing approximately 80% identity (allowing for conservative amino acid changes; Steward 1987).

Apart from these structural similarities, there may be analogous mechanisms regulating the activity of the proteins: the differences in the subcellular location of Rel in different cell types is reminiscent of the regulation of dorsal and NF-κB activity, which is dependent on their nuclear localization. In the inactive form, NF-κB is bound to another protein, Iκb which retains NF-κB in the cytoplasm. After inducing cells with mitogens or the tumor promoter TPA, Iκb is phosphorylated and released from the complex, resulting in nuclear translocation of NF-κB (Baeuerle and Baltimore 1988; Ghosh and Baltimore 1990). In the case of dorsal, which plays a crucial role in the formation of the dorsal-ventral axis, a nucleocytoplasmic gradient is observed in the embryo, i.e. a greater relative amount of nuclear dorsal protein is found in the ventral as opposed to the dorsal region. Although a cytoplasmic inhibitor analogous (and perhaps homologous) to Iκb has not been definitively identified, a candidate fulfilling this purpose is in sight – the *cactus* gene.

Transformation by v-Rel, however, seems to be largely independent of its intracellular location, since targetting of the protein to the nucleus by addition of a heterologous signal sequence does not significantly affect its transforming properties in lymphoid cells (Gilmore and Temin 1988). The fact that both cytoplasmic and nuclear v-Rel proteins can induce transformation is puzzling, but might suggest that transcriptional activation is not a prime mechanism involved. This observation may also point to different

mechanisms that can induce transformation by v-Rel. In spite of the structural similarities with dorsal and NF-κB, the mechanism of Rel-induced transformation remains an enigma at present. It appears, however, that the Rel family is another example of structurally similar transcriptional regulators whose function is modulated by the combinatorial association with other proteins (Ballard et al. 1990).

The v-Rel protein differs from its normal cellular homolog by the fusion with 13 *env*-derived amino acids, the deletion of 2 N-terminal and 118 C-terminal amino acids and the presence of 18 heterologous C-terminal amino acids due to an out-of-frame deletion (Capobianco et al. 1990; Hannink and Temin 1989). Although expression of v-*rel* from a retroviral vector leads to transformation of lymphoid cells, expression of c-*rel* in a similar vector does not result in transformation (Hoelzer et al. 1980; Hannink and Temin 1991). These observations indicate that certain structural differences between c-Rel and v-Rel are crucial for the induction of transformation by the v-*rel* gene. Deletion analyses suggest that alterations at either the N-terminus or the C-terminus of c-Rel protein are required and sufficient for the activation of its transformation potential (Hannink and Temin 1989; Bhat and Temin 1990). Which functions are encoded in these regions of the protein? Both regions may represent transcriptional activators, the C-terminal one is missing in v-Rel. There is, however, some controversy as to whether the N-terminus of Rel is an activator (Kerr et al. 1990) or a modulator of the C-terminal activator domain (Bull et al. 1990).

6 *myc:* Putative Transcriptional Regulator Involved in the Control of Cell Proliferation, Differentiation, and Tumorigenesis

The c-*myc* gene product represents another intranuclear mediator of the cellular response to growth factors (Armelin et al. 1984; Kaczmarek et al. 1985; Kelly et al. 1983; Rapp et al. 1985). The c-*myc* mRNA levels increase rapidly and transiently after treatment of different cell types with proliferation-inducing components (Kelly et al. 1983) following the induction of c-*fos* and *fos*B-L. In contrast to c-*fos*, c-*myc* mRNA is expressed at readily detectable levels at all stages of the cell cycle as well as in proliferating asynchronous cells, suggesting that the c-Myc product may play a role in cell cycle control (Hann et al. 1985; Hann and Eisenmann 1985; Rabbitts et al. 1985; Thompson et al. 1985) rather than having a specific function in the recruitment of quiescent cells. The apparent correlation of c-*myc* expression with proliferation is supported by the finding that the inhibition of HeLa cell proliferation by tumor necrosis factor or interferon is asso-

ciated with an inhibition of c-*myc* transcription (Yarden and Kimchi 1986). It has, however, to be pointed out that this correlation is not absolute, since terminally differentiated cells expressing high levels of c-*myc* have been identified (Hirvonen et al. 1990).

Quiescent mouse fibroblasts briefly exposed to platelet-derived growth factor (PDGF) or fibroblast growth factor (FGF) become competent to synthesize DNA (Stiles et al. 1979). The addition of platelet-poor plasma lacking PDGF allows these cells to progress through G_1 and enter the S phase (Stiles et al. 1979). The observation that induction of c-*myc* and c-*fos* expression is among the earliest events following growth factor stimulation led to their definition as competence genes. This is in agreement with the observation that c-Myc protein, either microinjected or expressed from an inducible transfected construct, partially abrogates the PDGF requirement of quiescent mouse fibroblasts for growth (Armelin et al. 1984; Kaczmarek et al. 1985).

Expression of c-*myc* does not only appear to be associated with proliferation but also seems to be inversely correlated with differentiation: c-*myc* is expressed at high levels in the human myeloid leukemia cell line HL60, but is switched off when the cells are induced by dimethylsulfoxide to differentiate to granulocytes (Bentley and Groudine 1986; Westin et al. 1982b). Likewise, in teratocarcinoma cells induced to endodermal differentiation with retinoic acid (Campisi et al. 1984; Dony et al. 1985) and in Friend cells induced to erythrocytic differentiation by dimethylsulfoxide c-*myc* expression is switched off (Lachmann and Skoultchi 1984). The apparent incompatibility of c-*myc* expression with terminal differentiation in most cell types is also suggested by the observation that its constitutive expression prevents differentiation (Coppola and Cole 1986; Prochownik and Kukowska 1986). The potential of c-Myc to severely interfere with differentiation and to perturb the control of cell proliferation probably explains the necessity for a great number of regulatory mechanisms acting at the level of transcription initiation, transcript elongation, and mRNA stability (Spencer et al. 1990).

Oncogenic activation of c-*myc* requires its deregulation, but no structural changes to the coding sequences: c-Myc protein can trigger the immortalization of rodent cells, it cooperates with cytoplasmic oncogenes in the induction of a fully transformed phenotype in primary rat fibroblasts (Land 1986), and it causes morphological transformation in chicken fibroblasts. Certain amino acid substitutions, however, as they occur in the viral proteins, enhance transformation (Patschinsky et al. 1986). These observations made with cloned *myc* genes reintroduced into suitable recipient cells are in agreement with the elevated and deregulated (constitutive) expression of c-*myc* found in chemically or spontaneously transformed cells

(Campisi et al. 1984). Alteration of the c-*myc* gene in tumor cells, presumably associated with its oncogenic activation, is achieved by several different molecular mechanisms. These include the deregulation of c-*myc* expression by (a) retroviral insertion, leading to the juxtaposition of the c-*myc*-coding region to the viral transcriptional control elements in avian and feline leukemias, (b) chromosomal translocations that bring the c-*myc* gene under the control of an immunoglobulin enhancer as in many cases of Burkitt's lymphoma (Haluska et al. 1983); and (c) amplification of the c-*myc* gene in human tumor cells, such as small cell lung carcinoma and neuroblastoma (reviewed by Zimmermann and Alt 1990). The oncogenic action of c-Myc has also been investigated in transgenic mice (Steward et al. 1984; Adams et al. 1985; reviewed by Cory and Adams 1988). These studies showed that the expression of exogenous *myc* alleles led to the development of a variety of tumors, which, however, occurred after certain latency periods and were of monoclonal origin. This suggests that deregulated c-*myc* expression can trigger tumor induction, but is insufficient on its own; additional alterations such as the sporadic activation of proto-oncogenes or inactivation of tumor suppressor genes are needed. This conclusion is confirmed by the finding that coexpression of c-*myc* and v-Ha-*ras* in transgenic mice results in a dramatic, synergistic acceleration of tumor formation (Sinn et al. 1987). These tumors are still monoclonal, indicating that even two oncogenes are insufficient for the full development of neoplasia.

Phosphorylation of the Myc protein occurs at both threonine and serine residues in two different regions (Ramsay et al. 1982; Bister et al. 1987). One of these domains is the central acidic domain in Myc which shows homology to known transactivation domains present in other transcription factors; this domain is, however, not required for transformation by v-Myc (Bister et al. 1987). In addition, phosphorylation also occurs at residues immediately adjacent to a basic domain located at the C-terminus (see below). The threonine and serine residues in both these domains are potential candidates for phosphorylation by casein kinase II (Lüscher et al. 1989), but the role of phosphorylation in this region is unknown.

An interesting picture has emerged for the structure and function of the last exon in Myc, exon 3, where a number of motifs indicative of sequence-specific DNA-binding proteins and transcriptional regulators have been identified. These motifs include an amphipathic helix-loop-helix motif (HLH), a basic domain and a leucine heptad repeat, all of which have now been shown to play an important role in DNA-protein as well as protein-protein interactions in other regulatory proteins such as Jun, Fos, E12/E47 and MyoD (Predergast and Ziff 1989). Analysis of the Myc protein has identified a region necessary for its interaction with DNA and for oligo-

merization of v-Myc, as lying within an 86 amino region, stretching from the basic motif and extending to the C-terminus (Kerkhoff and Bister 1991). The leucine heptad repeat and/or the HLH is essential for homodimerization of Myc and thus also for its ability to bind DNA specifically (Dang et al. 1989; Nakajima et al. 1989; Crouch et al. 1990; Kerkhoff et al. 1991). These domains are presumably also important for the interaction of Myc with other proteins, for example the recently identified HLH protein Max/Myn (Blackwood and Eisenman 1991; Prendergast et al. 1991). The arrangement of the different motifs within the Myc protein are similar to that found within two known transcription factors, USF and TFE3, which specifically bind the μE3 motif located in the adenovirus major late promoter and in the immunoglobulin heavy chain enhancer, respectively (Beckmann et al. 1990; Gregor et al. 1990). All three proteins possess the identical arrangement of motifs: basic domain-HLH-leucine repeat. Myc, TFE3, and USF not only share a common arrangement of motifs, but all three proteins also bind an identical palindromic DNA sequence, CACGTG, although the role of the flanking nucleotides in protein-DNA interaction remains to be investigated (Blackwell et al. 1990; Prendergast and Ziff 1991; Kerhoff et al. 1991). It has, however, yet to be shown that Myc is indeed a bona fide transcription factor and its cellular targets remain to be identified. Progress along these lines will also be a prerequisite to elucidate the exact mechanisms leading to Myc-induced transformation, in particular they answer the question as to whether transformation by Myc involves the transcriptional deregulation of other genes.

7 *myc*N and *myc*L: *myc* Family Members Identified in Human Tumor Cells

Two other *myc* family members *myc*N and *myc*L genes were identified by virtue of their homologies to the c-*myc* gene and their amplification in certain human tumors. Amplification of the *myc*N genes is found in different types of neuroectodermal tumors including gliomas, neuroblastomas, small cell lung tumors, retinoblastomas, and astrocytomas. There is a significant correlation between the degree of *myc*N amplification and the progression of neuroblastomas and thus the prognosis of the disease (Brodeur et al. 1984), such that the amplification of this gene can be used as a diagnostic tool. Amplification of *myc*N occurs within DNA sequences which vary in length between 100–1500 kbp. The amplified DNA is often arranged in a head-to-tail configuration known as homogeneously staining regions (Amler and Schwab 1989).

Like c-*myc*, both *myc*N and *myc*L cooperate with activated ras to transform nonestablished rat embryo fibroblasts (Land 1986; Schwab et al. 1985; Yancopoulos et al. 1985; DePinho et al. 1987), and *myc*N is able to rescue primary cells from senescence (Schwab and Bishop 1988). It has also been shown that *myc*N and *myc*N induce the formation of lymphomas in transgenic mice (Dildrop et al. 1989; Rosenbaum et al. 1989; Möröy et al. 1990). In agreement with these observations it has been found that *myc*N is activated in mouse T-cell lymphomas caused by Moloney murine leukemia virus (van Lohuizen et al. 1989; Dolcetti et al. 1989; Setoguchi et al. 1989).

Both the MycN and MycL proteins are located in the nucleus and undergo posttranslational modifications similar to c-Myc. All three proteins contain regions of homology: a region in exon 2 known as the Myc box (Nau et al. 1985), and in exon 3 the basic domain-HLH-leucine repeat. The functional significance of these domains in the Myc-related proteins has, however, yet to be shown. The pattern of expression of *myc*N and *myc*L is unlike that of c-*myc*, and is both spatially and temporally restricted. The level of expression of *myc*N and *myc*L in the newborn mouse is highest in the kidney, brain, and intestine (Zimmermann et al. 1986). Later in development, the level of expression of both *myc*-related genes falls dramatically in the forebrain, hindbrain, and kidneys. In the adult human brain, the levels of *myc*N gene expression is very low, but expression in neuroblastoma cells is comparable to that found in the fetal brain. These observations suggest that different *myc* genes may serve distinct functions in development and growth control, the *myc*-related genes presumably having cell type- and tissue-specific tasks. The functions of their encoded products in transcriptional regulations and their precise role in transformation remain, however, subjects of future investigations.

8 *myb:* Transregulator of the Myelomonocytic Lineage?

The v-*myb* gene was initially isolated from two retroviruses: avian myeloblastosis virus (AMV) and avian leukemia virus E26. Although the structure of the Myb fusion gene products encoded by the two viruses differs, both contain the central part of the c-*myb* gene (AMW: amino acids 72 to 442 of *myc*; E26: amino acids 81 to 363 of v-*myc*; Klempnauer et al. 1982, 1983; Nunn et al. 1983; Gerondakis and Bishop 1986). A high level of constitutive c-*myb* expression is restricted to the various lineages of the hematopoietic system, the highest levels being found in immature cells (Gonda et al. 1981; Westin et al. 1982a; Sheiness and Gardinier 1984), but

induction of c-*myb* is observed following serum stimulation of fibroblasts (Thompson et al. 1986). In agreement with the former observation, the induction of differentiation of immature cells by various agents has been shown to result in a fall in c-*myb* expression (Westin et al. 1982a; Gonda and Metcalf 1984). In addition, a high level of c-*myb* expression can block differentiation of hematopoietic cells, and antisense oligonucleotides to c-*myb* mRNA interfere with hematopoietic cell proliferation, suggesting that c-*myb* indeed plays a role in maintaining active proliferation and preventing differentiation of these cell types. The identification of a Drosophila c-*myb* homolog which is expressed throughout embryonal tissue development (Katzen et al. 1985) would suggest that the c-Myb protein does not just play an important role during hematopoiesis but also in cell proliferation in general. Another interesting feature of the c-*myb* gene is the alternative splicing of its transcripts, which generates a truncated protein, termed Mbm2, besides the full-length product c-Myb (Weber et al. 1990). While c-Myb blocks the dimethylsulfoxide-induced differentiation of mouse erythroleukemia cells, the shorter Mbm2 protein, which lacks the transcriptional regulatory regions, enhances their differentiation (Weber et al. 1990).

Both the viral and cellular form of Myb protein are phosphorylated at serine and threonine residues and have short half-lives of 30 min. In vitro they bind DNA specifically and have been shown to recognize the sequence PyAAC$^{G}/_{T}$G (Biedenkapp et al. 1988; Ness et al. 1989; Weston and Bishop 1989); whether or not the cellular and viral proteins differ in their in vivo binding is at present not known. It has been shown, however, that phosphorylation of residues lying at the N-terminus regulates the DNA-binding activity of the c-Myb protein and that these amino acids are absent from v-Myb (Lüscher et al. 1990). The transactivation of gene constructs containing Myb binding sites by c-*myb* and v-*myb* has been shown in different cell types and thus appears not to be cell type specific as transformation. To date, only the chicken *mim*-1 gene has been found to be specifically regulated by Myb (Ness et al. 1989), but since this gene is not activated by AMV v-Myb it is unlikely that mim-1 is essential for Myb-induced transformation. The mechanisms involved in Myb-induced transformation therefore remain poorly understood.

9 *ets:* A Multigene Family of Putative Transcriptional Regulators Affecting AP-1 Dependent Transcription

The v-*ets* is one of the two oncogenes of avian leukemia virus E26 where it seems to be responsible for the erythroleukemogenic potential of the

virus. Deletion of v-*ets* from the viral polyprotein abolishes induction of erythroblastosis but does not affect myeloid transformation, the latter being induced by the second oncogene of E26, *myb* (Nunn and Hunter 1989). Apart from its direct cellular progenitor c-*ets*-1 (Chen 1988; Watson et al. 1988), the cellular genome contains several other genes showing significant structural similarities in the region of the DNA-binding site, including c-*ets*-2 (Ghysdael et al. 1986; Boulukos et al. 1988; Watson et al. 1988), *erg* (Reddy et al. 1987; Rao et al. 1987), *elk*-1 (Rao et al. 1989), *elk*-2 (Rao et al. 1989), and Pu-1, which is probably identical with the putative oncogene *spi*-1, a common viral integration site in murine erythroleukemias (Morean-Gachelin et al. 1988). Ets-1, Ets-2 and Pu-1 have been shown to be sequence-specific DNA-binding proteins. A consensus sequence for Ets-1- and Ets-2-DNA binding has been derived by comparing binding sites identified in different promoters and by studying the effect of base changes. This consensus sequence C A/C GGAAG T/C (Günther et al. 1990; Bosselut et al. 1990; Wasylyk et al. 1990b) is different from the structurally related Pu box element, GAGGAA, the binding site of Pu-1 (Klemsz et al. 1990).

c-Ets-1, c-Ets-2 and Pu-1 possess transcription activating properties. Ets-1 and Ets-2 have been shown to transactive constructs consisting of the long terminal repeats (LTR) of the human T-cell leukemia virus HTLV-1 or multimerized versions of the polyoma virus PEA3 motif linked to the CAT gene (Bosselut et al. 1990; Wasylyk et al. 1990b). Likewise, Pu-1 can transactivate a reporter plasmid containing multimerized Pu-box elements (Klemsz et al. 1990). In the polyoma virus B element a TRE overlaps the Ets-binding site, and Ets-1 and Ets-2 have been found to cooperate with Jun/Fos in transcriptional activation (Wasylyk et al. 1990b). Interestingly, Ets-1 represses the human T-cell receptor gene by binding to a TRE located in its enhancer (Prosser et al. 1991). At the moment, the mechanism by which Ets proteins interact with DNA elements and other transcription factors remains largely elusive.

Both c-Ets-1 and c-Ets-2 are transiently induced and phosphorylated in response to an intracellular increase in Ca^{2+} or the activation of protein kinase C (Pognonec et al. 1990). In the case of Ets-1, serine phosphorylation leads to the loss of its nonspecific DNA-binding properties and a reduced affinity for chromatin, suggesting that phosphorylation may have a regulatory function (Pognonec et al. 1989; Boulukos et al. 1990). It is possible that Ets proteins act as transcription factors, subunits of transcription complexes and/or modulatory proteins whose expression and activity are regulated by extracellular signals triggering specific signal transduction pathways.

10 *erb*A: Oncogenic Activation Converts a Thyroid Hormone Receptor into a Transcriptional Repressor

The *erb*A gene was initially identified as one of the oncogenes encoded by the erythroblastosis virus AEV-ESA which induces sarcomas and leukemias in chickens (reviewed in Graf and Beug 1983). The v-*erb*A gene is expressed as a gag fusion protein $p75^{gag\text{-}v\text{-}erbA}$ (Hayman et al. 1979), which shares 45% amino acid homology with the DNA-binding site of steroid hormone receptors (Hollenberg et al. 1985; Krust et al. 1986; Weinberger et al. 1985). The cellular homolog, which is located on human chromosome 17 (Dayton et al. 1984; Spurr et al. 1984; Zabel et al. 1984), was shown to be identical with the receptor for thyroid hormone (Sap et al. 1986; Weinberger et al. 1986). In addition, there are two other closely related genes (Jansson et al. 1983), both located on human chromosome 3 (Weinberger et al. 1986). One form is primarily expressed in the brain and pituitary while the other two, including c-*erb*A, are expressed at low levels in a wide variety of cell types.

The v-ErbA protein has very little oncogenic potential alone both in vivo in chicks and in vitro in bone marrow cells and fibroblasts (Frykberg et al. 1983; Sealy et al. 1983). v-ErbA has, however, the potential to cooperate with a range of other oncogenes to enhance their oncogenic potential (for references see Zenke et al. 1990). For example, the expression of v-*erb*A permits the growth of transformed erythroblasts in normal culture medium (Kahn et al. 1986), most probably as the result of the inhibition of anion transporter (band 3) expression. The coexpression of v-*erb*A with v-*erb*B transformation defective mutants, or with normal c-*erb*B (EGF receptor), results in transformation (Damm et al. 1989; Jansson et al. 1987). While v-*src* expression induces sarcomas, coexpression with v-*erb*A results in the rapid and fatal development of erythroleukemias in chicks (Kahn et al. 1986). In addition, v-*erb*A is able to block the differentiation of erythroblasts transformed with growth-promoting oncogenes (Beug et al. 1985; Frykberg et al. 1983; Kahn et al. 1986).

In vitro expressed c-ErbA binds both triiodothyronine (T3) and thyroxine (T4) with affinities identical to those obtained for nuclear thyroid hormone receptors (Sap et al. 1986; Weinberger et al. 1986). In contrast, the viral form has lost the ability to bind hormone (Munoz et al. 1988), although it retained the sequence-specific DNA-binding properties (Damm et al. 1989; Sap et al. 1989). Both c-ErbA and v-ErbA contain two conserved cysteine-rich zinc fingers which are required for the sequence-specific binding to DNA. Nucleotide sequence analysis has revealed several amino acid differences between the two proteins (Sap et al. 1986), some of which are responsible for the different properties of the two forms of the protein.

There are a total of 13 amino acid substitutions in v-*erb*A, two of which are located in the DNA-binding domain. Whether these substitutions influence the DNA-binding specificity of the protein is at present not known. In v-ErbA, there is a deletion of 9 amino acids located 3 amino acids before the end of the C-terminus, a region which in c-ErbA has the potential to form an amphipathic helix. The analysis of v-*erb*A/c-*erb*A hybrid constructs suggests that the deletion of these amino acids is partially responsible for the loss of hormone-binding activity of the viral protein and is thus critical for oncogene activation (Zenke et al. 1990), and that the internal point mutations also contribute.

Using synthetic thyroid hormone responsive elements linked to reporter genes it has been shown in transient assays that in the absence of hormone T3, c-ErbA acts as a repressor of transcription. This inhibition is reversed in the presence of hormone where activation of transcription occurs. However, v-ErbA which is unable to bind hormone acts as a constitutive repressor of transcription and is capable of antagonizing c-ErbA function (Damm et al. 1989; Sap et al. 1989). It is believed that c-ErbA is always bound to DNA but that the binding of hormone induces a conformational change which results in the activation of transcription. v-ErbA, which no longer has the ability to act as a hormone-inducible activator of transcription, acts as a dominant transcription repressor (Damm et al. 1989). This transdominant negative function of v-ErbA may play a major role in its ability to potentiate the transforming properties of other oncogene products. To date it has been shown that v-*erb*A is able to specifically block the transcription of two erythrocyte genes: anion transporter (band 3) and ∂-aminolevulinic acid synthetase. The deregulation of these genes may be a crucial step in the potentiation of transformation by v-ErbA: it is conceivable that the inhibition of the band 3 anion transporter changes the intracellular pH with the consequence of triggering signal transduction pathways effecting cell division, while the block of hemin synthesis due to the repression of ∂-aminolevulinic acid synthetase transcription may prevent terminal differentiation.

11 Transcription Factor-Derived Oncoproteins: Common Mechanisms of Oncogenic Activation?

One of the most intriguing questions regarding the transforming potential of transcriptional regulators concerns the mechanism(s) by which oncogenic conversion is brought about. In the past few years, we have learned a great deal about the function of "nuclear proto-oncogenes" in the pro-

cess of transcriptional regulation and about the structural changes that discern the well-behaved proto-oncogenes from their deregulated (and deregulating) counterparts. But we were also taught that the mechanisms by which structurally altered transcriptional regulators cause transformation is considerably more complex than one might have expected (see the corresponding sections for references). There is no doubt that oncogenic conversion is functionally different for different genes: it seems that the loss of a repressor domain, the δ region, and perhaps the loss of a negative-regulatory phosphorylation site are important steps in the oncogenic activation of c-Jun, suggesting that transactivation is crucial. On the other hand, the ability of v-ErbA to cooperate with other oncogenes in the induction of neoplasia seems to be attributable to the loss of its transactivating potential, i.e., its conversion to a transdominant repressor. A similar mechanism seems to apply to v-Rel, which may also be a transdominant repressor or transcription due to the loss of a transactivation domain.

There is also evidence that more than one molecular mechanism may be involved in the induction of transformation by v-Rel, since the subcellular location apparently does not play a major role with regard to its transforming potential: both cytoplasmic and nuclear v-Rel transform lymphoid cells. This may suggest that the two forms of v-Rel protein utilize different mechanisms for the induction of transformation. The structural similarity with the subunits of NF-κB may provide some hints in this direction: it is possible that the cytoplasmic form interacts with and thus perhaps titrates a cytoplasmic protein (analogous to the association of NF-κB with I-κB), while the nuclear form may carry out some function involving DNA binding. This hypothesis will be testable when the molecular properties of c-Rel and v-Rel proteins are characterized in more detail.

For the induction of transformation by Fos, a rather complex picture has emerged. While it is clear that transrepression of SREs plays no significant if any role in the induction of transformation, the relevance of transactivation is less clear. Although a good correlation between the two functions exists for many mutants tested, this does not hold for others. This includes a N-terminal truncation of 110 amino acids, which destroys the ability of Fos to induce foci in fibroblasts, but which has only a marginal effect on transactivation. This discrepancy is emphasized by the observation that, at least when tested in an in vitro transcription assay, this deletion eliminates a transcription-repressing region. Two explanations are possible: (1) TRE-dependent transcription is not the (or not the major) target in Fos-induced transformation. The fact that the TIE is also a site of interaction with Fos protein complexes has already pointed out that other DNA recognition sequences exist and thus represent potential targets. (2) Transformation by Fos may involve more than one mechanism.

A candidate along these lines is the repression of the glucocorticoid and perhaps other steroid hormone receptors, which appears to be dependent on N-terminal sequences in Fos protein. It cannot be ruled out either that totally different functions of Fos (and Jun) may be involved in the induction of transformation, such as a role in DNA replication.

To add another feature to this complexity, many nuclear oncoproteins physically interact with one another and form, for instance, bipartite DNA-binding and transactivation domains, as it has been shown for Fos and Jun. In the case of Ets, effects on AP-1 elements have been described which apparently can affect transcription either positively or negatively, depending on the context of the promoter. It is possible that an understanding of the interaction of nuclear oncoproteins with one another and other cellular proteins may be the key to unraveling the mechanisms leading to the induction of neoplastic transformation by "nuclear oncogenes". In this context, the recent identification and cloning of a gene encoding a protein that interacts with Myc gene products, the *max/myn* gene, deserves particular attention, as it may finally provide a handle to understand the function of an oncogene that appears to play a major role in human tumorigenesis.

Acknowledgements. We are grateful to Dr. T. Möröy and D. Mumberg for critical reading of this paper and constructive criticism and to I. Kaminski for help in preparation of the manuscript. The work in the authors' laboratory is supported by the Deutsche Forschungsgemeinschaft (SFB215/D8 and Mu601/5-1) and the Dr. Mildred Scheel Stiftung für Krebsforschung.

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