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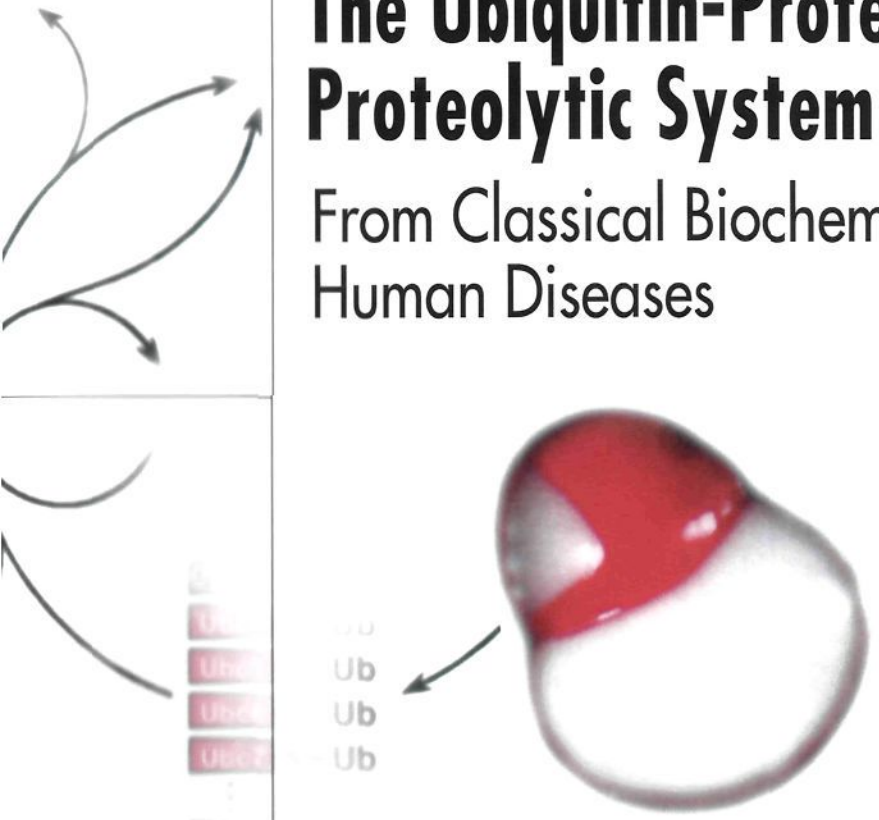
The Ubiquitin-Proteasome Proteolytic System

From Classical Biochemistry to Human Diseases



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The Ubiquitin-Proteasome Proteolytic System

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THE UBIQUITIN-PROTEASOME PROTEOLYTIC SYSTEM

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Preface

The finding that cellular proteins are turning over—synthesized and degraded constantly—has traversed a torturous road from its discovery in the 1940s until it has reached its current central position as a major regulatory pathway. The dynamic state of the proteome was discovered by Rudolph Schoenheimer who used radiolabeled compounds to demonstrate that proteins are in a constant state of generation and destruction (1). Yet, the extent, the mechanisms and the physiological significance of roles of protein degradation have remained elusive for many years. Simpson reported that degradation of labeled proteins in liver slices requires metabolic energy (2). This—thermodynamically paradoxical finding—where investment of energy is still required for the degradation of energy-rich macromolecules—proteins—to low energy small molecules—amino acids—has been corroborated in many studies ever since in both eukaryotes and prokaryotes. Since proteolysis is an exergonic process, the requirement for energy had remained an enigma. Simpson tried to explain that "*The fact that a supply of energy seems to be necessary for both the incorporation and the release of amino acids from protein might well mean that the two processes are interrelated*". He concluded however by saying that "*...the fact that protein hydrolysis as catalyzed by the familiar proteases and peptidases occurs exergonically, together with the consideration that autolysis in excised organs or tissue minces continues for weeks, long after phosphorylation or oxidation ceased, renders improbable the hypothesis of the direct energy dependence of the reactions leading to protein breakdown*" (2). The basic principle that cleavage of a peptide bond is exergonic, has not and could not have been challenged. Yet, the simple notion that proteases cannot exist in one compartment with their substrates without an energy barrier separating them, along with the high specificity of the process as we currently know it, makes energy investment in an

apparently exergonic process something we all accept now. Yet, the road to this acceptance has been long.

The discovery of the lysosome by Dr. Christian de Duve (reviewed in Ref. 3) has resolved some of the enigmas. Since the lysosome contains many acidic proteases, it was suggested that it must play a role in the degradation of intracellular proteins. Mortimore demonstrated a direct correlation between accelerated protein degradation that follows deprivation of nutrients to perfused liver, and increased lysosomal autophagy that is accompanied by a variety of structural alterations in the lysosomal system. Both the accelerated degradation and the structural changes could be reversed by re-supplementation of amino acids and hormones or serum (see for example Ref. 4). It was found that energy is required for activity of the lysosomal membrane proton pump that maintains the low intralysosomal pH necessary for optimal activity of the proteases (5). Different lines of experimental evidence along with the development of specific inhibitors strongly suggested that multiple pathways are involved in intracellular protein degradation, and the lysosome plays a role only in certain aspects of this process. Proteins were classified into short- and long-lived (reviewed in Refs. 6,7), but their different stability could not be explained based on the known mechanism of action of the lysosome that involves micro- and macroautophagy. During this process, entire droplets of cytosol and even subcellular organelles are engulfed with all the contained proteins digested at similar rates. While it was clear that lysosomal proteases are neither selective nor specific, certain studies still attempted to attribute specificity to lysosomal degradation. According to one model for example, all cellular proteins are engulfed into the lysosome, but only short- lived proteins that are sensitive to lysosomal proteases are degraded, whereas the more resistant, long-lived proteins escape back into the cytosol (8). The development of specific inhibitors of lysosomal proteases and of lysosomotropic agents—weak bases such as chloroquine or ammonium chloride—that are entrapped within the lysosome and increase the pH, thus inactivating lysosomal proteases—enabled researchers to examine in more detail the existence of distinct proteolytic pathways. Knowles and Ballard (9) and Neff and colleagues (10) demonstrated that leupeptin, antipain and chymostatin—specific lysosomal protease inhibitors—inhibit selectively degradation of long-lived but not of short-lived and abnormal proteins.

Poole and colleagues demonstrated that the lysosomotropic agent chloroquine selectively inhibits enhanced protein breakdown induced in cultured cells by depletion of serum, but has no effect on the degradation of cellular proteins under basal metabolic conditions (11). In an extremely elegant experiment, yet ingenious in its simplicity, he showed that chloroquine does not inhibit the degradation of endogenous cellular proteins that were metabolically labeled with ^{3}H-leucine, but at the same time and in the same ^{3}H-leucine-labeled cells, strongly inhibits the degradation of either endocytosed BSA, or endocytosed soluble cellular proteins that were prepared from identical cells metabolically labeled with ^{14}C-leucine (11). He concluded that intracellular proteins degraded under stress, or endocytosed/pinocytosed extracellular proteins are degraded within lysosomes following their engulfment from the cytosol or transfer from the cell membrane to the lysosome along the vacuolar system, respectively. In contrast, under basal metabolic conditions, intracellular proteins, and in particular short-lived ones, are degraded by a yet unidentified non-lysosomal system(s) (reviewed in Refs. 7 and 12).

To identify and characterize this non-lysosomal system, Etlinger and Goldberg chose the reticulocyte as a model system. This cell lacks lysosomes and is involved in extensive degradation of its organelles and enzymatic systems prior to maturation in the bone marrow and conversion to a circulating erythrocyte. They found that the reticulocyte contains an ATP-dependent proteolytic system that degrades abnormal, amino acid analog-containing, short-lived proteins (13). Working in parallel, Hershko and Ciechanover fractionated the reticulocyte extract and found that a small—~8.0 kDa—heat stable protein is necessary to reconstitute proteolysis of a model substrate in a crude lysate from which it was first removed during fractionation (14). The protein was designated ***A***TP-dependent ***P***roteolysis ***F***actor-***1*** (APF-1), as it became clear that the system contains several additional factors that may act in concert. Mechanistic studies revealed that multiple moieties of APF-1 are covalently conjugated—in an ATP-dependent mode—to the substrate (15). This surprising finding led the two researchers, along with Rose, to propose a model according to which degradation of a protein via the system involves two steps (i) conjugation of multiple molecules of APF-1 to the substrate, and (ii) degradation of the tagged substrate with release of reusable APF-1 (16). Parallel studies

identified APF-1 as ubiquitin, a known protein of hitherto unknown function (17, 18). Ubiquitin was discovered by Goldstein and colleagues as a protein that induces differentiation of B and T cells, and is ubiquitously distributed in prokaryotes and eukaryotes, hence its name (19). Later analyses revealed that it is not involved in regulating lymphocytes development, and that prokaryotes do not express it. Yet, the descriptive name was retained. An interesting finding related to ubiquitin was the identification by Busch and colleagues of the nucleolar protein A24 (see for example Ref. 20). Structural analyses (21,22) revealed that A24 has a unique bifurcated structure in which ubiquitin is conjugated—in an isopeptide bond—via its C-terminal Gly^{76} to the ε-NH_2 group of Lys^{119} of histone 2A. The function of protein A24 has remained an unsolved mystery to our days. Yet, the finding that its level is decreased following hydrolysis to its two components, histone H2A and ubiquitin, during liver regeneration (23) or erythropoesis (24), as well as a later finding that it is associated preferentially to nucleosomes that are localized at the 5' end of actively transcribed genes (25), led to the hypothesis that it plays a role in transcriptional regulation. As noted, all the changes observed in the level of the protein involve its hydrolysis and re-assembly and not degradation and resynthesis. In light of our current understanding of the ubiquitin system, this is because proteins that are modified by a single moiety of ubiquitin cannot be recognized by the 26S proteasome, the protease of the system (see below) that degrades only multiply ubiquitinated substrates. Identification of APF-1 as ubiquitin and the known structure of A24 led to the hypothesis that the C-terminal Gly residue of ubiquitin must be activated prior to its conjugation in a mechanism that is enzymatically similar to the activation of amino acids by aminoacyl tRNA-synthetase during ribosome-based polypeptide synthesis, or to the activation of amino acids during ribosome-free oligopeptide biosynthesis (see for example Ref. 26). Indeed, experiments in fractionated extracts showed that intermediates similar to those generated during amino acid activation, are generated also during activation of ubiquitin (27). Using the deciphered activation of ubiquitin, Ciechnaover and Hershko used immobilized ubiquitin to purify, via mechanism-based "covalent" affinity chromatography and reversal of the activation reaction, the first enzyme in the ubiquitin pathway cascade, the ubiquitin-activating enzyme, E1 (28). Purification of the two other enzymes in the ubiquitin relay reaction, the

ubiquitin-carrier protein, E2 (later designated also the ubiquitin-conjugating enzyme, UBC), and the ubiquitin-protein ligase, E3 (29) followed shortly after. The many members of the E3 family bind the target substrates via defined motifs and endow the system with its high specificity. Generation of antibodies to ubiquitin allowed to demonstrate, for the first time, that the system is active also in nucleated cells *in vivo* and not only in the terminally differentiating reticulocyte—the model cell studied initially: a direct correlation was observed between levels of ubiquitin adducts and rates of abnormal protein degradation induced by incubation of the cells in the presence of amino acid analogues (30). Stronger and more direct evidence was later obtained by Varshavsky, Finley and Ciechanover, who characterized a known cell cycle arrest mutant that loses A24 at the non-permissive temperature. They identified the mutation as a thermolabile E1 that, when inactive, cannot re-conjugate ubiquitin to histone 2A (31). Heat inactivation of the enzyme leads to severe impairment in the degradation of short-lived abnormal proteins generated during incubation of the cells in the presence of amino acids analogs (32). The original observation by Yamada and colleagues that loss of the thermolabile E1 leads to arrest at the S/G2 phase, enabled the three researchers to predict that the ubiquitin system is required for cell cycle progression. This hypothesis was later corroborated by numerous studies demonstrating ubiquitin intermediacy in the degradation of many cell cycle regulators. Later studies led to the discovery that it is a polyubiquitin chain—in which the ubiquitin moieties are linked to one another—that generates the high molecular mass adducts and the proteolytic signal (33). Beforehand, a formal possibility still existed that the high molecular mass adducts represent multiple single moieties attached to distinct lysine residues. The chain is composed of ubiquitin moieties that are linked to one another via an isopeptide bond between the C-terminal Gly76 of one ubiquitin moiety and an internal Lys48 of the previously conjugated moiety (34). In parallel, the downstream protease—the 26S proteasome complex—was discovered (35-37), and the entire pathway could be studied in different experimental systems.

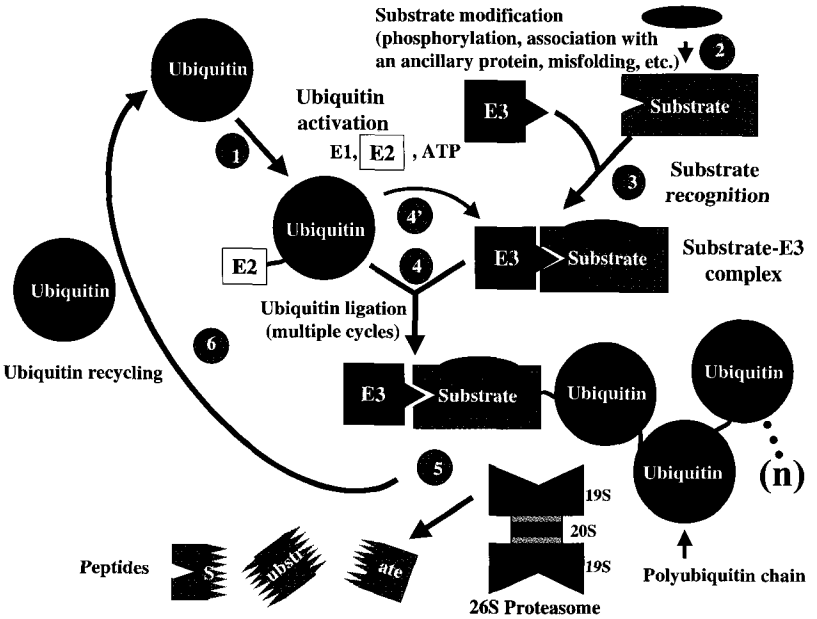
The first insights on the problem of specific substrate recognition began to emerge at that time. Using a biochemical approach, Hershko demonstrated that the N-terminal residue may play a role in recognition of certain model substrates (38), and that recognition is mediated via binding

of this residue to the ubiquitin ligase (39). Ciechanover showed that tRNA-Arg is required for the degradation of certain proteins (40) that have acidic N-termini. Arg-tRNA-protein transferase catalyzes a reaction that adds an Arg residue to the acidic N-terminal residue, and the conversion of the charge allows recognition of the substrate by the ubiquitin ligase E3α (41). Varshavsky used a genetic approach in yeast to generate 20 distinct species of a derivative of β-galactosidase that differed solely in the identity of the N-terminal residue. Yet, the stability of the proteins varied significantly, which led to the formulation of a rule, the N-end rule, according to which the identity of the N-terminal residue determines the stability of the protein (42). Later studies revealed that the N-terminal recognition signal contains adjacent lysine residues that serve as ubiquitination anchors (43). Recognition of the N-terminal residue cannot provide a general targeting mechanism since the N-terminal residue of most substrates is not accessible for recognition by the ligase as it is acetylated (44). We now know that recognition of proteins by the ubiquitin system is far more complex then originally thought. Proteins are targeted by multiple ligases following recognition of different primary and secondary motifs, post-translational modifications and recognition in *trans* mediated by association with ancillary proteins. Yet, the discovery of N-terminal recognition, the first targeting motif, was of great importance, as it drew attention to a centrally important problem—the requirement for specific recognition of the substrates.

These early studies did not change the prevailing view that the main role of the system is to rid the cell from mutated/misfolded/abnormal proteins and the research focus remained on the puzzle of how the system selectively recognizes and eliminates abnormal proteins, leaving intact their normal counterparts, when the differences can be minute and sometime indiscernible. It was not until the early 1990s—when researchers started to discover that specific key cellular proteins, such as transcriptional and cell cycle regulators, are targeted by the system in a regulated manner (see for example Refs. 45-49)—that we have begun to see an exponential growth in the number of published studies and a general recognition of the role of the system in basic cellular processes such as regulation of transcription, cell cycle progression, growth and differentiation, the immune and inflammatory responses, and quality control. Not too long after that researchers started to

realize that aberrations in such a complex pathway underlie the pathogenesis of many diseases, both inherited and acquired. The discoveries of ubiquitin-like proteins and their role in non-proteolytic functions such as routing of certain proteins to their subcellular destinations or protecting others from ubiquitination and destruction, of mono-ubiquitination and its role in regulating the endocytic pathway, and of polyubiquitin chains that involve residues other then Lys48 in transcriptional regulation, have expanded the scope of ubiquitin conjugation beyond degradation and set it in a centrally important position among other regulatory mechanisms. The evolution of two distinct regulatory mechanisms, phosphorylation that is reversible and proteolysis that is irreversible, has been inevitable evolutionarily. For certain processes such as cell cycle progression, the unidirectional movement along a "one way" road must be controlled in a tight manner. Like the wife of Lot that on her way from Gomorrah "*looked back from behind him and she became a pillar of salt*" (Bible, Torah, Genesis, 19, 26), the cell cycle cannot look or go back.

Where is research on the ubiquitin system heading now? Important knowledge is still missing on the specific function of the E3s and their substrates. It is likely that, based on the recognition of common structural motifs such as the HECT domains, RING fingers and U-boxes, the human genome will unravel hundreds of novel ligases and lead to the discovery of their substrates, the processes involved, and the aberrations caused by selective malfunction of these enzymes. Some of these enzymes will have auto-ubiquitinating activity that may serve as a regulatory, "self-destructive" mechanism, others will have both *cis* and *trans* activities. Does BRCA1 have specific substrates? Which processes are derailed by its mutation? These and many other questions still await answers. Resolving the 3D structure of the ligases with their substrate may aid in developing mechanism-based specific drugs that will interfere with specific processes. Protease inhibitors are already making their way as potential drugs against many diseases such as malignancies and immune and inflammatory disorders (see for example Refs. 50, 51), yet they clearly cannot be specific and rely for their activity on a narrow toxicity window. Drugs that target specific ligases will affect a narrower subset of substrates. Better and even more specific drugs may be those that will interfere specifically with the



The ubiquitin proteasome pathway. (1) ATP-dependent activation of ubiquitin by the ubiquitin activating enzyme, E1, and by a ubiquitin-carrier protein (ubiquitin-conjugating enzyme, UBC), E2, to generate a high-energy E2~ubiquitin intermediate. (2) Modification of the substrate (phosphorylation or oxidation, for example), its association with an ancillary protein (chaperone or a viral protein, for example) or its misfolding are required for its recognition and specific binding to the ubiquitin ligase, E3 (3). (4) Generation of a substrate-anchored polyubiquitin chain catalyzed by direct transfer of the ubiquitin moiety from the E2~ubiquitin complex to the E3-bound substrate (RING finger E3s). (4') Generation of a substrate-anchored polyubiquitin chain catalyzed by transfer of the ubiquitin moiety from the E2~ubiquitin complex to the E3 to generate an additional E3~ubiquitin high energy intermediate from which the activated ubiquitin moiety is transferred to E3-bound substrate [catalyzed by HECT (*H*omologous to *E*6-AP *C*-*T*erminus) domain E3s]. U-box-containing E3s have also been described, but their mechanism of function has not been discerned. (5) Degradation of the polyubiquitinated substrate by the 26S proteasome complex with release of free and reusable ubiquitin (6) catalyzed by ubiquitin recycling enzymes (ubiquitin C-terminal hydrolases; deubiquitinating enzymes, DUBs; isopeptidases).

interaction of substrates with ancillary exogenous proteins, such as p53-E6 interaction (52). Interference with endogenous ancillary proteins such as molecular chaperones may prove to be extremely toxic. An additional line of research will involve dissection of non-proteolytic functions of ubiquitin and ubiquitin-like proteins, the requirements for specific substrate recognition and the role of ligases in these processes. All this new knowledge will not only broaden our basic knowledge on the ubiquitin system, but will drive the system from the test tube to the patient bed.

This conference has told us the story of the ubiquitin system, as we currently know it, glowing and shiny. From regulation of basic cellular processes such as cell cycle progression and transcription, through quality control and the pathogenetic mechanisms of disease, from X-ray crystallography of the 26S proteasome, to the interaction between substrates and their ligases, to the development of mechanism-based drugs to target specific aberrant processes. But this is the epilogue. It started differently. The history of intracellular protein degradation is an illuminating example of a modern "*Cinderella*". She started her life in the garbage, literally, helping the cell cleaning it. Carving her way up the mountain, she taught us several important lessons. One is that cleaning garbage is a respected trade. Accumulation of mutated/misfolded/aggregated proteins underlies the pathogenesis of many diseases, including several neurodegenerative disorders such as Huntington's disease. Maintaining the steady state level of growth stimulators, such as β-catenin and HIF-1α, or tumor suppressors such as p53, is also essential. Accumulation of the first and accelerated degradation of the latter has been implicated in the pathogenesis of several malignancies. Finally, she taught us that normal proteins have to be destroyed as well: programmed destruction of cyclins allows cell cycle progression, whereas removal of transcriptional activators and their inhibitors, regulates specific gene expression. As King Solomon taught us thousands years ago "*To everything there is a season and a time to every purpose: A time to be born, and a time to die; a time to plant, and a time to pluck up that which is planted; a time to kill, and a time to heal; a time to break down, and a time to build up*" (Bible, Hagiograph, Ecclesiastes, 3, 1–3).

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Contents

The Ubiquitin System and Some of Its Roles in Cell Cycle Control

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The selective degradation of many short-lived proteins in eukaryotic cells is carried out by the ubiquitin-mediated proteolytic system. In this pathway, proteins are targeted for degradation by covalent ligation to ubiquitin, a highly conserved small protein. The ligation of ubiquitin to protein involves the successive action of three types of enzymes: the ubiquitin-activating enzyme E1, a ubiquitin-carrier protein E2 and a ubiquitin-protein ligase, E3. The selectivity and the regulation of the degradation of a specific protein are usually determined by the properties of its specific ubiquitin ligase (E3) enzyme. Recently we have been studying two ubiquitin ligase complexes that have important roles in different aspects of cell cycle regulation. One is the cyclosome, or Anaphase-Promoting Complex (APC), which acts on mitotic cyclins and some other regulators in exit from mitosis. The cyclosome is activated at the end of mitosis by phosphorylation, a process that allows its further activation by the ancillary protein Cdc20. A different complex, which belongs to the SCF (Skp1-Cullin-F-box protein) family of ubiquitin ligases, is involved in the degradation of p27, a mammalian G1 cyclin-dependent kinase (Cdk) inhibitor, following mitogenic stimulation. Its action is triggered by Cdk2-dependent phosphorylation of p27, as well as by the increase in levels of a specific F-box protein Skp2 and of the cell cycle regulatory protein Cks1, that all take place in the G1 to S-phase transition.

For many years, I have been interested in the problem of how proteins are degraded in cells. The dynamic state of cellular proteins (Schoenheimer, 1942) and the important roles of protein degradation in the control of cellular enzyme levels (Schimke & Doyle, 1970) have been recognized for a long time, but the underlying molecular mechanisms remained unknown. A clue to an unusual mechanism was provided by observations indicating that the

degradation of cellular proteins requires metabolic energy (Simpson, 1953; Hershko & Tomkins, 1971). In 1978, biochemical fractionation in my laboratory of an ATP-dependent cell-free proteolytic system from reticulocytes showed that a small, heat-stable protein was required for its activity (Ciechanover *et al.*, 1978). In 1980, we have found that this protein (subsequently identified as ubiquitin) is covalently ligated to protein substrates, and proposed that ubiquitin ligation targets proteins for degradation (Hershko *et al.*, 1980). Our further fractionation-reconstitution work has shown (Hershko *et al.*, 1983) that ubiquitin-protein ligation involves the sequential action of three enzymes. First, a ubiquitin-activating enzyme, E1, uses the energy of ATP hydrolysis to form a thiolester bond with ubiquitin. Next, activated ubiquitin is transferred to a ubiquitin-carrier protein, E2. Finally, ubiquitin is transferred to the protein substrate by the action of a ubiquitin-protein ligase, E3 (Hershko *et al.*, 1983). Further work by several laboratories has shown that there is a single E1, but there are multiple species of E2s and E3s, involved in the ligation to ubiquitin of different proteins. The selectivity of protein degradation is mainly determined by the specificity of the binding of a certain class of cellular proteins to a specific E3 enzyme. Proteins ligated to polyubiquitin chains are degraded by the 26S proteasome complex and free ubiquitin is released by the action of ubiquitin-C-terminal hydrolases or isopeptidases (reviewed in Hershko & Ciechanover, 1998).

These studies on the basic biochemistry of the ubiquitin pathway were all carried out in the reticulocyte sytem, using artificial protein substrates, such as extracellular proteins or denatured proteins. Though many gaps remained in our understanding of the basic biochemistry of the ubiquitin system, about ten years ago I thought that it was important at this stage to turn to the question of how the degradation of some specific cellular proteins is carried out by the ubiquitin system in a selective and regulated fashion. This is how I became interested in the roles of the ubiquitin system in the cell division cycle, because the levels of many important cell cycle regulatory proteins rise and then fall in the cell cycle (reviewed in Hershko, 1977). Here I describe briefly our more recent work on the mode of the degradation of two different cell cycle regulators: cyclin B, the major mitotic cyclin, and p27, an inhibitor of cyclin-dependent kinases in animal cells.

Cyclin B is the positive regulatory subunit of protein kinase Cdk1. It was the first cyclin discovered, as a protein that is destroyed at the end of each cell cycle in early embryos of marine invertebrates, such as sea urchins

and clams (Evans *et al.*, 1983). In 1991, independent work by Glotzer *et al.* (1991) and from our laboratory (Hershko *et al.*, 1991) has indicated that cyclin B is degraded by the ubiquitin system. Both laboratories have employed biochemical approaches, using cell-free systems from early embryonic cells that faithfully reproduce the selectivity and regulation of cyclin B degradation *in vitro*. With a cell-free system from *Xenopus* eggs, Glotzer *et al.* (1991) have shown that cyclin B is degraded and is ubiquitinylated only in mitosis, but not in the interphase. We found that in extracts of fertilized clam oocytes, the degradation of both cyclin A and cyclin B was inhibited by methylated ubiquitin, a derivative of ubiquitin that blocks the formation of polyubiquitin chains (Hershko *et al.*, 1991).

The above-described studies have suggested that the degradation of mitotic cyclins is carried out by the ubiquitin pathway, but they have not identified the components responsible for the specificity and regulation of cyclin degradation. For this purpose, the fractionation of extracts and the isolation of the active components was required. Initial fractionation was achieved by our laboratory (Hershko *et al.*, 1994). Fractionation of extracts of clam oocytes showed that in addition to E1, two novel components were required to reconstitute cyclin-ubiquitin ligation *in vitro*. These were a specific E2, called E2-C, and an activity associated with particulate material. Though E2-C specifically acts on this system, and homologues of E2-C are present in many (though not all) eukaryotic organisms (reviewed in Hershko, 1997; Townsley & Ruderman, 1998), its activity is not regulated in the cell cycle. By contrast, the activity of the component associated with particulate material was cell cycle regulated: it was inactive in the interphase, and became active in mitosis (Hershko *et al.*, 1994).

This slow start has paved the way to rapid progress in our knowledge of this system that took place in 1995. This progress was due to convergence of information from biochemical work with genetic analysis in yeasts. In a work done in collaboration with Joan Ruderman, we have dissociated the cell cycle-regulated component from the particulate fraction oocytes and have partially purified and characterized it (Sudakin *et al.*, 1995). It was found to be a large (~1,500 kDa) complex that has cyclin-ubiquitin ligase activity. The activity of the isolated complex remained cell cycle regulated. Furthermore, the inactive complex from interphase cells could be converted *in vitro* to the active form by incubation with protein kinase Cdk1/cyclin B. We have called this complex the cyclosome, to denote its large size and important roles in cell cycle regulation (Sudakin *et al.*, 1995). A similar

biochemical approach of fractionation of *Xenopus* egg extracts by King *et al.* (1995) has identified a similar complex that was termed Anaphase-Promoting Complex, or APC. The identification of the subunits of the cyclosome/APC was made possible by the work of Irniger *et al.* (1995), who have used an elegant screen to isolate yeast mutants defective in cyclin B proteolysis. Vertebrate homologues of the products of some of these genes were shown to be subunits of the *Xenopus* cyclosome/APC (King *et al.*, 1995). Further work has identified 10–12 subunits of the cyclosome/APC in yeast and in higher eukaryotes (reviewed in Zachariae & Nasmyth, 1999). It thus seems that the cyclosome/APC is highly conserved in evolution, from from yeast to clams to humans.

The activity of the cyclosome/APC is tightly regulated in the cell cycle. In early embryonic cells, a basal regulatory mechanism is the reversible phosphorylation of the cyclosome/APC. We found that the active, mitotic form of the clam cyclosome is inactivated by incubation with an okadaic acid-sensitive phosphatase. Addition of protein kinase Cdk1/cyclin B to phosphatase-treated cyclosome preparation restored activity (Lahav-Baratz *et al.*, 1995). More recent genetic and biochemical work in other laboratories has identified two WD-40 repeat containing proteins, called Cdc20 and Cdh1, that are required for the activity of the cyclosome/APC in the M and G1 phases of the cell cycle, respectively (reviewed in Zachariae and Nasmyth, 1999). We found that the phosphorylation of the cyclosome is required for its activation *in vitro* by Cdc20 (Shteinberg *et al.*, 1999), thus explaining the M-phase specific action of this ancillary factor. Similar results were obtained in a recent genetic study in yeast (Rudner & Murray, 2000). Thus, both *in vitro* and *in vivo* evidence indicate an inter-relationship between protein phosphorylation and protein degradation in the control of exit from mitosis: the protein kinase Cdk1/cyclin B activates the ubiquitin ligase cyclosome/APC by its phosphorylation, and the cyclosome/APC subsequently inactivates the protein kinase by the degradation of its cyclin B subunit. Other substrates, functions and regulatory mechanisms of the cyclosome/APC are discussed in an excellent review of Zachariae and Nasmyth (1999).

The second project on which I have been working recently, in collaboration with Michele Pagano, is the mode of the degradation of $p27^{Kip1}$. p27 is an inhibitor of mammalian G1 cyclin-dependent kinases such as Cdk2/cyclin E, which is responsible for driving cells from G1 to the S-phase of the cell cycle (reviewed in Sherr & Roberts, 1999). Levels of p27

are high in quiescent cells, thus inhibiting the action of Cdk2. Following stimulation of cells to grow p27 is rapidly degraded, coincident with a rise in levels of cyclin E. The degradation of p27 is thus essential to allow the action of Cdk2/cyclin E to drive cells into the S-phase. It has been previously shown that p27 is degraded by the ubiquitin system (Pagano *et al.*, 1995). We asked the question which ubiquitin ligase targets p27 for degradation and how is its action regulated in the G1 to S-phase transition. Here again, I have used a cell-free system that faithfully reproduces cell cycle stage-specific ubiquitinylation of p27 in the test tube, this time a cell-free system from cultured Hela cells (Montagnoli *et al.*, 1999). Again we have used bichemical fractionation, except that in this case we could try to guess the nature of the ubiquitin ligase involved, based on some previous bits of information. It was known that the phosphorylation of p27 on Thr187 by Cdk2 is required for its degradation *in vivo* (Sheaff *et al.*, 1997; Vlach *et al.*, 1997), and for its ubiquitinylation *in vitro* (Montagnoli *et al.*, 1999). In many cases, substrate phosphorylation is required for its recognition by an SCF (Skp1-Cullin-F-box protein) type of ubiquitin ligase (reviewed in Deshaies, 1999). These ubiquitin ligase complexes contain several constant components and a variable subunit, called an F-box protein, that recruits specific protein substrates for ubiquitinylation. Because of the requirement of p27 ubiquitinylation on its phosphorylation, we suspected that an SCF-type ubiquitin ligase may be involved. We have therefore subjected extracts of HeLa cells to immunodepletion with antibodies directed against mammalian F-box proteins and found that immunodepletion of the F-box protein Skp2 (S-phase kinase-associated protein 2) completely abolished p27-ubiquitin ligation activity. Activity could be completely restored by the supplementation of purified recombinant Skp2. These *in vitro* findings, together with *in vivo* results of the Pagano laboratory, established that p27 is targeted for degradation by an SCF complex which contains Skp2 as its specific F-box protein (Carrano *et al.*, 1999). Similar conclusions were reported by other investigators (Sutterluty *et al.*, 1999; Tsvetkov *et al.*, 1999). However, an important piece of the puzzle was missing, since we could not reconstitute p27-ubiquitin ligation with known purified components of the SCF^{Skp2} complex. Using biochemical fractionation and purification, we have recently identified the missing component as Cks1 (cyclin kinase subunit 1), a protein which belongs to the Suc1/Cks family of cell cycle regulatory proteins (reviewed in Pines, 1996). Cks1 reconstitutes p27-ubiquitin ligation in a completely purified system, binds to Skp2 and

greatly increases the binding of T187-phosphorylated p27 to Skp2 (Ganoth *et al.*, 2001). Similar conclusions were reached in an independent study of Spruck *et al.* (2001), using Cks1 knockout mice. These findings indicate that an accessory protein is required for the activity of an SCF complex and for its binding to a phosphorylated substrate. The results also show that the degradation of p27 in the G1 to S transition is subject to several levels of regulation. It has been shown that levels of Skp2 are very low in G0/G1, and increase greatly before entry into the S-phase and the expression of Cks1 is similarly regulated in the cell cycle (see Ganoth *et al.*, 2001 and references therein). Thus, in the G1 to S-phase transition, p27 is first phosphorylated by the increase of Cdk2/cyclin E levels, and is then targeted for degradation by a ubiquitin ligase complex that assembles at this stage from newly synthesized Skp2 and Cks1 components. Such multiple levels of regulation allow a tight control of this important cell cycle transition. For further information on the mechanisms and regulation of p27 degradation, the reader is referred to the chapter by Bloom and Pagano.

As I have pointed out previously (Hershko, 1996; Hershko *et al.*, 2000), the main lesson from my story is the continued importance of biochemistry in current biomedical research. The biochemical fractionation of the cell-free ATP-dependent proteolytic system from reticulocytes allowed the discovery of the role of ubiquitin ligation in protein degradation and the identification of the main enzymatic components of this pathway. More recently, extracts from clam oocytes and from *Xenopus* eggs, that faithfully recapitulate cell cycle-related events in the test tube, were instrumental in the identification of the cyclosome/APC, an important player in cell cycle control. Most recently, extracts of HeLa cells were used to define the components of the SCF/Skp2/Cks1 system that targets p27 for degradation, an event necessary for entry of mammalian cells into the S phase. I would also like to point out that biochemistry is most useful when combined with molecular genetics. In the ubiquitin story, the use of molecular genetics was essential in uncovering the widespread roles of this system in basic cellular processes such as cell cycle control, signal transduction, development and the immune and inflammatory responses.

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The Ubiquitin System and the N-End Rule Pathway

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Summary

Eukaryotes contain a highly conserved multienzyme system which covalently links a small protein ubiquitin (Ub) to a variety of intracellular proteins that bear degradation signals recognized by this system. The resulting Ub-protein conjugates are degraded by the 26S proteasome, an ATP-dependent multisubunit protease. Pathways that involve Ub play major roles in a huge variety of processes, including cell differentiation, cell cycle, and responses to stress. In this paper, I briefly review the design of the Ub system, describe in more detail one of its pathways, called the N-end rule pathway, and consider three recent discoveries: the finding that ubiquitin ligases interact with specific components of the 26S proteasome (1), the finding that peptides accelerate their uptake into cells by activating the N-end rule pathway (2), and the finding that the degradation of a cohesin subunit by the N-end rule pathway is essential for chromosome stability (3).

The Ubiquitin System

Ubiquitin (Ub) is a 76-residue protein that exists in cells either free or covalently linked to other proteins. Ub-dependent pathways have been shown to play major roles in a legion of biological processes, including cell

differentiation, cell cycle, embryogenesis, apoptosis, signal transduction, DNA repair, transmembrane and vesicular transport, stress responses (including the immune response), and functions of the nervous system (4-9). Many (but not all) of the Ub-dependent pathways involve processive degradation of Ub-conjugated (ubiquitylated) proteins by the 26S proteasome, an ATP-dependent multisubunit protease (10, 11).

Ub-specific enzymes catalyze reactions whose product is either a single Ub moiety or a multi-Ub chain covalently linked to an acceptor protein (Fig. 1). Ub is conjugated to other proteins through an amide bond, called the isopeptide bond, between the C-terminal (Gly-76) residue of Ub and the ε-amino group of a Lys residue in acceptor proteins (4, 5). Ub is activated for conjugation by a Ub-activating enzyme (E1), which couples ATP hydrolysis to the formation of a high-energy thioester bond between Gly-76 of Ub and a specific Cys residue of E1 (Fig. 1). The E1-linked Ub moiety is transferred, in a transesterification reaction, from E1 to a Cys residue of a Ub-conjugating enzyme (E2), and from there to a Lys residue of an ultimate acceptor protein, yielding a Ub-protein conjugate (Fig. 1) (6). This last step requires participation of another component, called E3, which selects a protein substrate for ubiquitylation through interaction with a substrate's degradation signal. E3s of at least one class, exemplified by a mammalian E3 called E6AP, are also enzymes whose specific Cys residues serve as acceptors of Ub from an associated E2 enzyme. The E3-linked Ub moiety is then conjugated to a Lys residue of an ultimate substrate (7).

Complexes of specific E2s and E3s catalyze the formation of multi-Ub chains linked to proteins targeted by the Ub system. The term "Ub ligase" is used below to denote either a specific E3 or an E3-E2 complex. In multi-Ub chains, the C-terminal Gly of one Ub is joined to an internal Lys of the adjacent Ub moiety, resulting in a chain of Ub-Ub conjugates in which the number of Ub moieties varies from 2 to many (12, 13). Since Ub has 7 lysines, a multi-Ub chain can have, *a priori*, a huge number of different topologies (connectivity patterns), depending, for example, on whether only one Lys residue of a Ub moiety is Ub-linked, and whether it is the same Lys residue in all Ub moieties of a chain. The first multi-Ub chain to be discovered had its Ub moieties conjugated through the Lys-48 residue of Ub (12). Other *in vivo*-formed multi-Ub chains involve Lys-63 or Lys-29 of

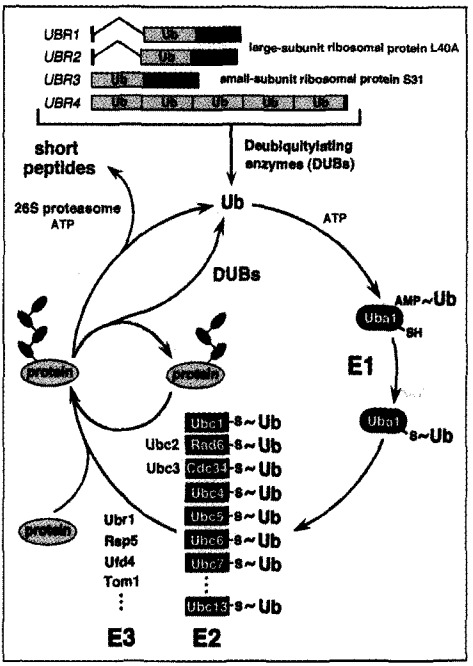

Figure 1. The ubiquitin (Ub) system of *S. cerevisiae*. The yeast Ub genes, two of which (*UBI1* and *UBI2*) contain introns, encode fusions of Ub (yellow rectangles) to itself (*UBI4*) or to one of the two specific ribosomal proteins (*UBI1-UBI3*) (red and blue rectangles) (42). These fusions are cleaved by deubiquitylating enzymes (DUBs), yielding mature Ub (17). Thioester bonds between Ub and the active-site Cys residues of Ub-specific enzymes are denoted by the ~ sign. The conjugation of Ub to other proteins involves a preliminary ATP-dependent step, in which the last (Gly-76) residue of Ub is joined, via a thioester bond, to a Cys residue in the Ub-activating (E1) enzyme encoded by the *UBA1* gene. The activated Ub is transferred to a Cys residue in one of at least 13 distinct Ub-conjugating (E2) enzymes encoded by the *UBC*-family genes, and from there to a Lys residue of an ultimate acceptor protein (yellow oval). This last step, and apparently also the formation of a multi-Ub chain (black ovals) require participation of another component, called E3. The function of E3 includes, but is not limited to, the recognition of a degradation signal in the acceptor protein (see the main text). The names of some of the currently known yeast E3's (Ubr1p, Rsp5p, Ufd4p, and Tom1p) are indicated as well. "Rad6" and "Cdc34" refer to the alternative (earlier) names of the Ubc2p and Ubc3p E2 enzymes. A targeted, ubiquitylated protein substrate is processively degraded to short peptides by the ATP-dependent 26S proteasome (4-6).

Ub. A multi-Ub chain linked through Lys-63 functions in DNA repair and the cell cycle-regulated modification of the ribosome (14-16). As discussed below, one function of a substrate-linked multi-Ub chain is to facilitate the substrate's degradation by the 26S proteasome.

The isopeptide bond between Ub and other proteins can be hydrolyzed: there are multiple, ATP-independent proteases (the yeast *Saccharomyces cerevisiae* has at least 20 of them) whose common property is the ability to recognize Ub moiety and cleave at the Ub-adduct junction. One cause of the striking multiplicity of these deubiquitylating enzymes (DUBs) (17) is the diversity of their targets, which include linear (DNA-encoded) Ub fusions, Ub adducts with small nucleophiles such as glutathione, and also free and substrate-linked multi-Ub chains.

The N-End Rule Pathway

This pathway of the Ub system was identified in 1986, through the discovery of the first degradation signal (degron) in short-lived proteins (18, 19). Degrons are features of proteins that confer metabolic instability (20). Some of these features are understood better than others, and new degrons are being discovered. Degradation signals can be active constitutively or conditionally. Signals of the latter class-found in many regulators, including cyclins and transcription factors-are controlled through phosphorylation or interactions with other proteins, whose binding may sterically shield an otherwise constitutive degradation signal (21).

The degradation signal of the N-end rule pathway, termed the N-degron, was identified through the invention of the Ub fusion technique, which made it possible to produce a desired residue at the N-terminus of a test protein in a cell (18). The *in vivo* half-life of a protein was found to depend on the identity of its N-terminal residue, a relation termed the N-end rule. The N-degron, a signal recognized by the N-end rule pathway, comprises two essential determinants: a destabilizing N-terminal residue and an internal lysine (or lysines) of a substrate (22, 23). In *S. cerevisiae*, there are two classes of destabilizing residues, basic, or type 1 (Arg, Lys and His) and bulky hydrophobic, or type 2 (Phe, Leu, Tyr, Trp and Ile). Ubr1p, a 225 kD

RING-H2 finger-containing E3, directly recognizes these N-terminal residues (19). A complex of the E3 Ubr1p and the E2 enzyme Rad6p (Ubc2p) mediates the synthesis of a multi-Ub chain linked to a Lys residue of the substrate (24). Subsequently identified degradation signals appear to be organized similarly to the N-degron, in that they comprise two distinct elements, an amino acid sequence or a conformational determinant (analogous to a destabilizing N-terminal residue) and a Lys residue or residues, the latter being the site of ubiquitylation (21).

The mechanistically and functionally complex N-end rule pathway (Fig. 2) is but one of many distinct pathways of the Ub system. The vast functional range of this system stems from the enormous diversity of its physiological substrates. In other words, it is the constitutive or conditional degradation of many specific proteins (cyclins, transcription factors, components of signal transduction pathways, damaged proteins) by ubiquitin-dependent pathways that underlies the involvement of the Ub system in just about every biological circuit in a living cell (5, 6, 9).

Already a decade ago, the Ub field was almost too large for a detailed description in a single review. Such a task is impossible nowadays. In this paper, I will illustrate some of the functional and mechanistic aspects of the Ub system by describing three recently published results. One of them uncovered specific physical interactions between the targeting (E3) and proteolytic (proteasome) components of the Ub system (1). The other finding deepened the understanding of the N-end rule pathway, and at the same time provided the first example of regulation of the Ub system through an allosteric interaction of E3 with small compounds (2). The third result (produced through a collaboration between Dr. H. Rao in my laboratory and Dr. F. Uhlmann in the laboratory of Dr. K. Nasmyth) revealed the essential role of the N-end rule pathway in maintaining the fidelity of chromosome segregation (3).

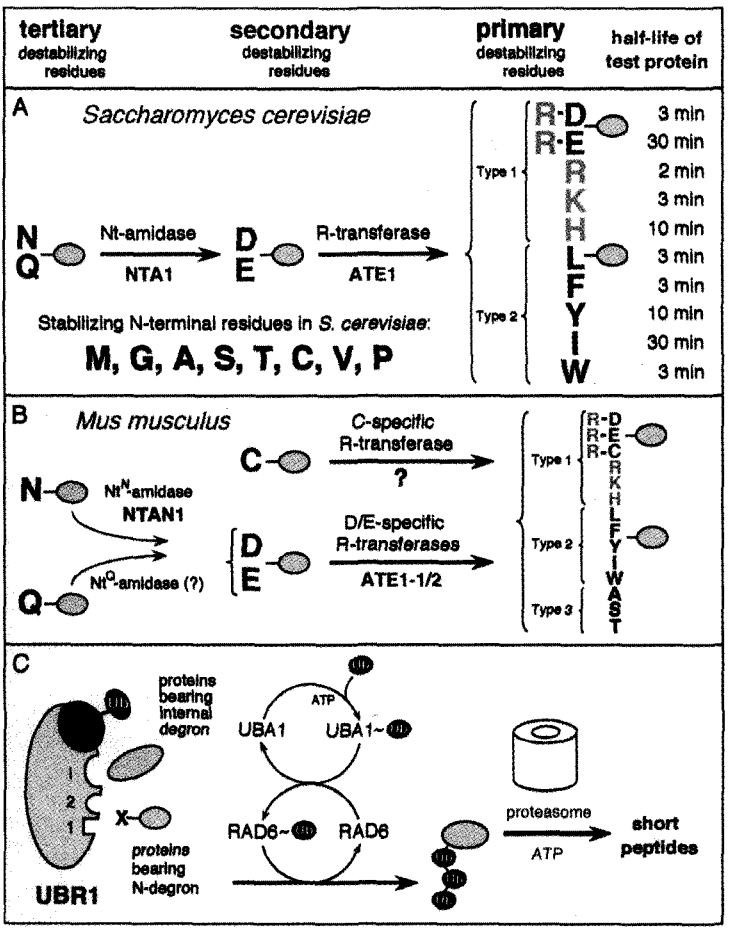

Figure 2. The N-end rule pathway in yeast (**A**) and the mouse (**B**). The type 1 and type 2 primary destabilizing N-terminal residues are in purple and red, respectively. Secondary and tertiary destabilizing N-terminal residues are in light blue and green, respectively. The yellow ovals denote the rest of a protein substrate. In **A**, the *in vivo* half-lives of X-βgals, β-galactosidase-based test proteins bearing different N-terminal residues in *S. cerevisiae* (19), are shown on the right. Stabilizing residues are in black. X-βgal proteins bearing stabilizing N-terminal residues are metabolically stable ($t_{1/2}$ > 20 hr). The conversion of tertiary destabilizing residues **N** (Asn) and **Q** (Gln) into secondary destabilizing residues **D** (Asp) and **E** (Glu) is mediated by N-terminal amidohydrolase (Nt-amidase), encoded by *NTA1* (43). The conjugation of the primary destabilizing residue **R** (Arg) to the secondary destabilizing residues **D** and **E** is mediated by Arg-tRNA-protein transferase

(R-transferase), encoded by *ATE1*. In the mammalian N-end rule pathway (**B**), the deamidation step is mediated by two distinct enzymes, Nt^N-amidase and Nt^Q-amidase, which are specific, respectively, for N-terminal Asn and Gln (44). In vertebrates, the set of secondary destabilizing residues contains not only Asp and Glu but also Cys (C), which is a stabilizing residue in yeast (34, 45). The mammalian counterpart of the yeast R-transferase ATE1 exists as two distinct species, ATE1-1 and ATE1-2, which are produced through alternative splicing of *Ate1* pre-mRNA (46). In mammals but in yeast, Ala (A), Ser (S) and Thr (T) are primary (type 3) destabilizing residues (19). (**C**) *S. cerevisiae* UBR1 has two binding sites for the primary destabilizing N-terminal residues of either proteins or short peptides. The type 1 site is specific for the basic N-terminal residues Arg, Lys and His. The type 2 site is specific for the bulky hydrophobic N-terminal residues Phe, Leu, Trp, Tyr and Ile. UBR1 contains yet another substrate-binding site, denoted as "i" (internal), which targets proteins bearing internal (non-N-terminal) degrons. In yeast, these proteins include CUP9 (2). A complex of UBR1 and the Ub-conjugating (E2) enzyme RAD6 produces a substrate-linked multi-Ub chain (19). A multiubiquitylated substrate is degraded by the 26S proteasome (10, 11).

Physical Association of Ubiquitin Ligases and the 26S Proteasome

The ATP-dependent 26S proteasome, which processively degrades a targeted, ubiquitylated substrate, consists of the 20S core protease and two 19S particles. A 19S particle mediates the binding and unfolding of a substrate protein before its transfer to the interior of the 20S core (10, 11). It is unclear how a targeted substrate is delivered to the 26S proteasome, in part because Rpn10p (Mcb1p/Sun1p), the only known proteasomal subunit that binds multi-Ub chains (25), is not essential for degradation of many proteins in the yeast *Saccharomyces cerevisiae* (26) .

It was recently discovered that *S. cerevisiae* Ubr1p and Ufd4p, the E3 components of two distinct Ub-dependent proteolytic pathways, directly interact with specific proteins of the 26S proteasome (1). These results stemmed from the initial finding that overexpression of some subunits of the 19S particle inhibited ubiquitin-dependent degradation of engineered N-end rule substrates. To determine whether this effect could be caused by interaction of these subunits with a component(s) of the N-end rule pathway, glutathione transferase (GST)-pulldown assays with Ubr1p (also called

N-recognin), the pathway's E3, were carried out. The 225 kD Ubr1p targets proteins that bear certain (destabilizing) N-terminal residues (Fig. 2). Two substrate-binding sites of Ubr1p recognize two classes of destabilizing N-terminal residues, basic and bulky hydrophobic. Yet another substrate-binding site of Ubr1p targets proteins that bear internal (non-N-terminal) degrons (2, 27). Similar but distinct versions of the N-end rule pathway are present in all organisms examined, from prokaryotes to fungi and mammals (19).

In the GST-pulldown assays, Ubr1p was N-terminally tagged with the FLAG epitope. Rpt1p and Rpt6p, two subunits of the 19S particle, were expressed in *E. coli* as fusions to the C-terminus of GST (1). Extracts of *S. cerevisiae* overexpressing FLAG-Ubr1p were incubated with glutathione-agarose beads pre-loaded with GST-Rpt1p, GST-Rpt6p, or GST alone. The bound proteins were eluted, fractionated by SDS-PAGE, and immunoblotted with a monoclonal anti-FLAG antibody. Remarkably, GST-Rpt6p was found to bind FLAG-Ubr1p; a smaller but significant amount of FLAG-Ubr1p was also bound by GST-Rpt1p, whereas no FLAG-Ubr1p was bound by GST alone (Fig. 3A, lanes 2, 7, 8). Could Ubr1p interact with other proteins of the 26S proteasome as well? Using the same assay, it was found that GST-Rpn2p was also able to bind FLAG-Ubr1p, whereas all of the other tested proteasomal components (GST-Rpn1p, GST-Rpn3p, GST-Rpn10p, GST-Rpn11p, GST-Rpn12p, GST-Rpt2p, and GST-Pre6p, the latter a component of the 20S core proteasome) did not bind to FLAG-Ubr1p (Fig. 3A). Coomassie staining of the eluted, SDS-PAGE-fractionated proteins confirmed that the amounts of different GST fusions pre-bound to glutathione-agarose beads were approximately equal (data not shown).

To determine whether Ubr1p binds to Rpt1p, Rpt6p, and Rpn2p directly, the c pulldown assays were carried out with purified FLAG-Ubr1p (Fig. 3B). FLAG-Ubr1p was overexpressed in *S. cerevisiae*, and purified in three consecutive steps: anti-FLAG antibody affinity chromatography; Rad6p (E2) affinity chromatography; and gel filtration (F. Du, A. Webster, and A. Varshavsky, unpublished data). In agreement with the results obtained using extracts from cells overexpressing FLAG-Ubr1p (Fig. 3A), the purified FLAG-Ubr1p bound to the purified GST-Rpt1p, GST-Rpt6p, and GST-Rpn2p, but not to GST-Rpn1p, GST-Rpn10p, or GST alone

(Fig. 3B). Coimmunoprecipitation-immunoblotting assays were then used to examine the *in vivo* interaction between Ubr1p and Rpt6p. The results (1) indicated that ha-Rpt6p was specifically coprecipitated with FLAG-Ubr1p by anti-FLAG antibody. We also carried out the reciprocal co-immunoprecipitation assay, and found that FLAG-Ubr1p was specifically coprecipitated with ha-Rpt6p by anti-ha antibody. These and related data demonstrated that Ubr1p, the E3 of the N-end rule pathway, is physically associated with the 26S proteasome. Moreover, Ubr1p was found to interact with more than one protein of the 19S particle (Fig. 3A, B).

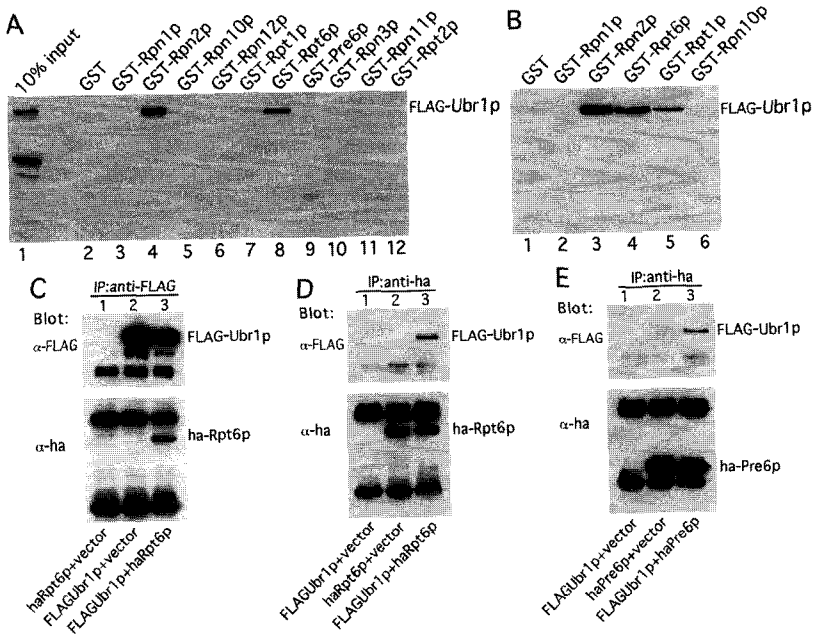

Figure 3. Ubr1p, the E3 of the N-end rule pathway, is physically associated with the 26S proteasome. (**A**) and (**B**), Ubr1p interacts with Rpn2p, Rpt1p and Rpt6p in GST-pulldown assays. Extracts of *S. cerevisiae* containing overexpressed FLAG-Ubr1p (**A**), or the purified FLAG-Ubr1p protein (**B**) were incubated with glutathione-agarose beads pre-loaded with the indicated GST fusions. The retained proteins were eluted, fractionated by SDS-8% PAGE, and immunoblotted with anti-FLAG antibody. Approximately equal amounts of different GST fusions were

immobilized on glutathione-agarose beads in these assays, as verified by Coomassie staining (data not shown). (**C**) and (**D**), *In vivo* association of Ubr1p and Rpt6p. Extracts of *S. cerevisiae* AVY107 (*ubr1*Δ) expressing either both FLAG-Ubr1p and ha-Rpt6p, or FLAG-Ubr1p alone, or ha-Rpt6p alone were incubated with anti-FLAG antibody (**C**) or anti-ha antibody (**D**). The immunoprecipitated proteins were separated by SDS-12% PAGE and transferred to nitrocellulose membrane. The top halves of **C** and **D** show the results of immunoblotting with anti-FLAG antibody; the bottom halves show the analogous data with anti-ha antibody. (**E**) Co-immunoprecipitation of Pre6p and Ubr1p. Extracts of *S. cerevisiae* AVY107 (*ubr1*Δ) expressing both FLAG-Ubr1p and ha-Pre6p, or FLAG-Ubr1p alone, or ha-Pre6p alone were incubated with anti-ha antibody, followed by the immunoprecipitation/immunoblotting described in **C** and **D** (1).

The substrates of another Ub-dependent proteolytic system, termed the UFD pathway (Ub/fusion/degradation) (28-30), include proteins bearing at their N-termini a "non-removable" Ub moiety. A partial or complete resistance of these Ub-containing proteins to DUB-mediated cleavage stems from either alterations of the last residue of Ub moiety or the presence of proline at the C-terminal side of the Ub-protein junction (29). Ufd4p, a member of the HECT family of E3 proteins, is the E3 of the *S. cerevisiae* UFD pathway. Sequence comparisons did not detect statistically significant similarities between the 225 kD Ubr1p and the 167 kD Ufd4p. GST-pulldown and coimmunoprecipitation-immunoblotting assays were used to determine whether the ha-tagged Ufd4p could also bind to the proteasome (1). In the GST-pulldown assay, extracts from *S. cerevisiae* overexpressing ha-Ufd4p were incubated with glutathione-agarose beads pre-loaded with GST-Rpn1p, GST-Rpn2p, GST-Rpn3p, GST-Rpn10p, GST-Rpn11p, GST-Rpn12p, GST-Rpt1p, GST-Rpt2p, GST-Rpt6p, or GST alone. The retained proteins were fractionated by SDS-PAGE and immunoblotted with anti-ha antibody. As shown in Figure 4A, ha-Ufd4p was found to be specifically bound to GST-Rpt6p, one of three proteins of the 19S particle that interacted with Ubr1p, the E3 of the N-end rule pathway. None of the other tested proteasomal proteins were able to interact with ha-Ufd4p in the GST-pulldown assay (Fig. 4A). These results, together with those of coimmunoprecipitation experiments (1), demonstrated that Ufd4p, the E3 of the UFD pathway, was physically associated with the proteasome.

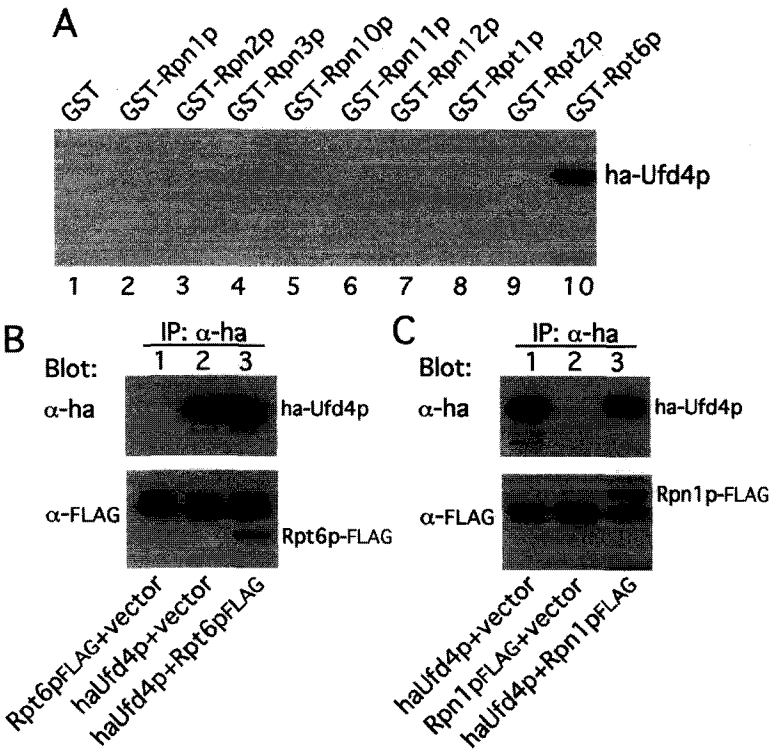

Figure 4. Ufd4p, the E3 of the UFD pathway, is physically associated with the proteasome. (**A**) Ufd4p interacts with Rpt6p in the GST-pulldown assay. Extracts of *S. cerevisiae* containing overexpressed ha-Ufd4p were incubated with glutathione-agarose beads pre-loaded with different GST fusions, as indicated. The retained proteins were eluted, fractionated by SDS-8% PAGE, and immunoblotted with anti-ha antibody. Approximately equal amounts of different GST fusions were immobilized on glutathione-agarose beads in these assays, as verified by Coomassie staining (data not shown). (**B**) *In vivo* association of Ufd4p and Rpt6p. Extracts of *S. cerevisiae* JD52 (*UBR1*) expressing both ha-Ufd4p and Rpt6p-FLAG, or ha-Ufd4p alone, or Rpt6p-FLAG alone were incubated with anti-ha antibody. The immunoprecipitated proteins were separated by SDS-10% PAGE and transferred to nitrocellulose membrane. The top half shows the results of immunoblotting with anti-ha antibody; the bottom half shows the data with anti-FLAG antibody. **C**, Co-immunoprecipitation of Rpn1p and Ufd4p. Extracts of *S. cerevisiae* JD52 expressing both ha-Ufd4p and Rpn1p-FLAG, or ha-Ufd4p alone, or Rpn1p-FLAG alone were incubated with anti-ha antibody, followed by the immunoprecipitation-immunoblotting procedure described in **B,** except that SDS-7% PAGE was used (1).

The earlier studies of Ub-binding components of the proteasome and the above results with E3 proteins (Figs. 3 and 4) indicate that two distinct elements of a targeted, Ub-conjugated substrate could serve as ligands for the substrate's docking at the 26S proteasome: a substrate-linked multi-Ub chain and/or a cognate E3-E2 complex reversibly bound to the substrate's degron. Thus far, Rpn10p is the only proteasomal component known to bind multi-Ub chains; it also binds to chain-bearing model substrates (25). However, *S. cerevisiae* strains lacking Rpn10p were found to be impaired in the degradation of only some of the model substrates, and did not exhibit aberrant phenotypes characteristic of proteolysis-impaired proteasome mutants (13, 26). It is possible that the apparent lack of absolute requirement for Rpn10p is caused by the mutual complementation between two mechanistically distinct ways of bringing a substrate to the proteasome: through the binding of a substrate-linked multi-Ub chain to Rpn10p or through the binding of a substrate-bound E3 to other specific subunits of the 19S particle. Another possibility is that there exists a different, functionally essential component of the 26S proteasome that binds to substrate-linked multi-Ub chains and remains to be identified.

The finding that Ubr1p and Ufd4p, the E3 components of, respectively, the N-end rule pathway and the UFD pathway, directly interact with specific proteins of the 26S proteasome suggests a general mechanism for the delivery of targeted substrates to the proteasome. In this model, the E3 component of a Ub-dependent pathway binds (as an E3-E2 complex) to a cognate degron of a target protein, and initiates, either sequentially or concurrently, two sets of processes: (i) The formation of a substrate-linked multi-Ub chain, through the activity of substrate-bound Ub ligase (E3-E2) complex. (ii) The delivery of an E3-bound substrate to the chaperone-like proteins of the 26S proteasome, through interactions between the E3 and specific proteins of the 19S particle. A key assumption of this model is that the demonstrated physical affinity between an E3 and the proteasome is essential for the normal level of activity of an E3-mediated proteolytic pathway. In the case of the N-end rule pathway, this prediction can be tested, for example, through the mapping of a site(s) in the 225 kD Ubr1p that mediates its binding to Rpn2p, Rpt1p and Rpt6p of the 19S particle. The site(s) thus identified could then be mutated in ways that leave intact the other functions of Ubr1p, such as its binding to substrates, to the Rad6p E2 enzyme, and the previously described, RING finger-dependent activity of

Ubr1p in the Rad6p/Ubr1p-mediated formation of a substrate-linked multi-Ub chain (31). A similar approach could be used to test the model's assumption in regard to the demonstrated Ufd4p-Rpt6p interaction.

Several aspects of the proposed delivery mechanism remain unconstrained by the available evidence. For example, if the Ubr1p-proteasome and Ufd4p-proteasome interactions (Figs. 3 and 4) prove relevant to the functional activity of the N-end rule and UFD pathways, one would like to determine how many of the distinct E3s in a cell interact with the proteasome, and what proteins of the proteasome these E3s bind to. Another important question is whether the Ubr1p-dependent formation of a substrate-linked multi-Ub chain is required, *in vivo*, for the docking of a Ubr1p-bound substrate at the Ubr1-binding site of the proteasome. An alternative model is that these two Ubr1p-mediated, substrate-centered processes (the multi Ub chain formation and the E3-mediated docking at the proteasome) take place concurrently and independently.

The previously proposed function of a substrate linked multi-Ub chain is to serve as a dissociation-slowing device (12). Specifically, if the rate-limiting step that precedes the first cleavages of a proteasome-bound substrate is the substrate's unfolding by chaperones of the 19S particle, then a decrease in the rate of dissociation of the proteasome-substrate complex, brought about by the multi-Ub chain, should facilitate substrate's degradation: the longer the allowed "waiting" time, the greater the probability of a required unfolding event (19). Similar considerations may apply to the function of E3-mediated binding of a substrate to the proteasome.

Detailed mechanisms of the Ub/proteasome-dependent multienzyme machines that target a protein substrate and then processively destroy it remain to be understood. The findings described above (1) identified a class of interactions between the targeting and proteolytic branches of the Ub system that does not involve a multi-Ub chain.

Peptide Import and the N-End Rule Pathway

The rate of degradation of specific proteins is often regulated by modulating the exposure or the structure of their degrons. For example, the degrons of

the cyclin-dependent kinase inhibitors Sic1p and p27 are activated by phosphorylation, which is timed to bring about their destruction at key transition points in the cell cycle (32). In other cases, phosphorylation regulates the activity of an E3 itself. For example, the anaphase-promoting complex (APC), a multisubunit E3, is activated only at mitosis (7).

Dipeptides bearing a destabilizing N-terminal residue of either basic or hydrophobic type act as competitive inhibitors of the degradation of N-end rule substrates carrying the same type of destabilizing residue (33-35). As described above, Ubr1p, the E3 of the N-end rule pathway, contains two distinct N-terminal residue-binding sites, called the type 1 and type 2 sites, that are each capable of binding either a dipeptide or a protein, but not both at the same time.

Recent work (2) identified the first physiological function of Ubr1p in *S. cerevisiae*: Ubr1p regulates the uptake of peptides (36) by controlling degradation of the 35 kD homeodomain protein Cup9p, a transcriptional repressor of the di- and tripeptide transporter Ptr2p (27). Ubr1p was found to target Cup9p through a degron located in the C-terminal half of Cup9p (F. Navarro-Garcia, G. Turner and A.Varshavsky, unpublished data), indicating that Ubr1p contains a third substrate-binding site, distinct from the type and type 2 sites. Despite this mode of Ubr1p-Cup9p interaction, we asked whether dipeptides bearing destabilizing N-terminal residues could affect the Ubr1p-mediated degradation of Cup9p, since dipeptides are able to inhibit degradation of canonical (N-degron-bearing) substrates.

To address this question, Turner et al. used Cup9pNSF, a C-terminally FLAG-tagged variant of the Cup9p repressor bearing an Asn-265 → Ser substitution. Cup9pNSF was degraded indistinguishably from wildtype Cup9p but was predicted to have a much lower affinity for DNA, so it would not influence the expression of Ptr2p and the uptake of dipeptides. Cup9pNSF was expressed as part of a fusion of the form FDHFR-Ub-Cup9pNSF (Fig. 5A). DUBs (17) cotranslationally cleave this UPR (Ub/protein/reference) fusion at the Ub-Cup9p junction, yielding the test protein Cup9pNSF and the long-lived FDHFR-Ub reference protein, which serves as an internal control for variations in expression levels and immunoprecipitation efficiency. (For descriptions of the UPR technique, see [Lévy et al., 1996 (47); Suzuki et al., 1999 (23)].)

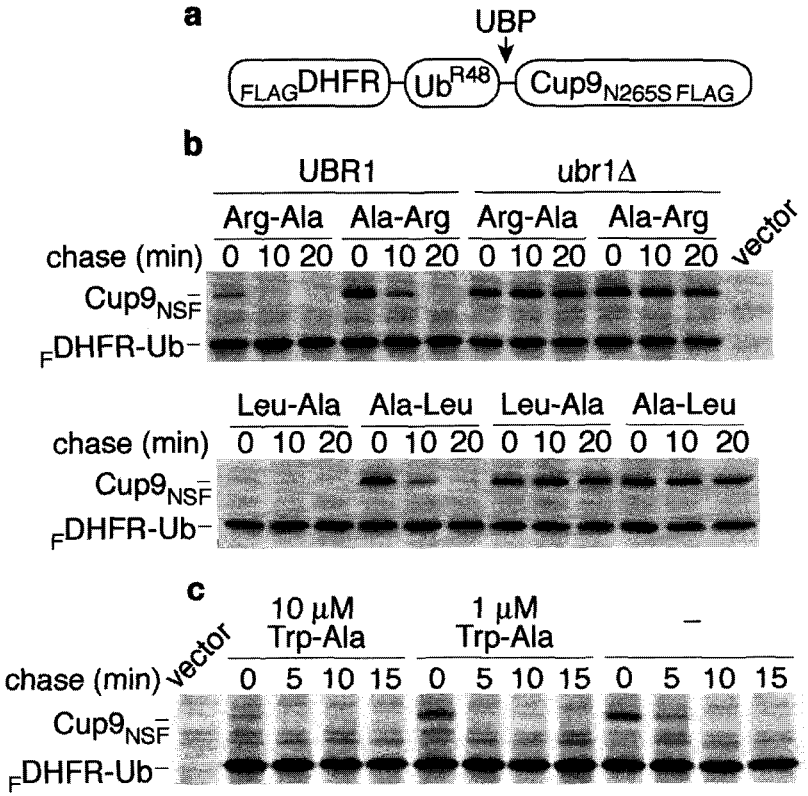

Figure 5. Enhancement of CUP9 degradation by dipeptides bearing destabilizing N-terminal residues. (**A**) The fusion protein used for pulse-chase analysis. The stable $_F$DHFR-Ub reference portion of the fusion is co-translationally cleaved from CUP9$_{NSF}$ by UBPs. (**B**) Pulse-chase analysis of $_F$DHFR-Ub-CUP9$_{NSF}$ in the presence of various dipeptides at 10 mM. Dipeptides bearing either basic (Arg-Ala) or bulky hydrophobic (Leu-Ala) destabilizing N-terminal residues strongly enhance CUP9$_{NSF}$ degradation, but only in strains expressing Ubr1p. Dipeptides bearing a stabilizing N-terminal residue (Ala-Arg and Ala-Leu) do not alter CUP9$_{NSF}$ degradation. **C**, The effects of different concentrations of Trp-Ala on the enhancement of CUP9$_{NSF}$ degradation in wildtype (*UBR1*) cells. Lanes marked by a dash indicate pulse-chase analysis performed in the absence of added dipeptides. Enhancement of CUP9$_{NSF}$ degradation was detectable at 1 μM Trp-Ala, and became substantially greater at 10 μM (2).

Cells expressing Cup9pNSF were grown in minimal medium containing allantoin as the nitrogen source to avoid the known effects of nitrogen catabolite repression on PTR2 expression. Leu-Ala and Arg-Ala, dipeptides bearing either type of destabilizing N-terminal residue (Leu, bulky hydrophobic; Arg, basic), were added to a final concentration of 10 mM. This dipeptide concentration results in maximal inhibition of degradation of N-end rule substrates (35). Strikingly, the addition of either Leu-Ala or Arg-Ala exerted an opposite effect on Cup9pNSF, strongly accelerating its degradation in wildtype (UBR1) cells. The half-life of Cup9pNSF decreased from ~5 min in the absence of dipeptides (Fig. 5C) to less than 1 min in their presence (Fig. 5B). This stimulatory effect was not observed in a ubr1Δ strain, indicating that the augmented degradation of Cup9pNSF was dependent on Ubr1p. The enhancement of degradation required dipeptides bearing destabilizing N-terminal residues: dipeptides of the same composition but bearing a stabilizing residue (Ala-Leu and Ala-Arg) did not affect the degradation of Cup9pNSF ($t_{1/2}$ ~5 min) (Fig. 5B and data not shown). Similar results were obtained with cells expressing Cup9pNSF that was not a part of a UPR fusion (data not shown).

To determine the concentration dependence of the stimulation, Turner et al. measured the degradation of Cup9p at a range of concentrations of Trp-Ala. The enhancement of Cup9pNSF degradation was detectable at 1 μM Trp-Ala, the lowest concentration tested ($t_{1/2}$ ~1 min) (Fig. 5C). In contrast, the degradation of Cup9pNSF was not altered either by Ala-Trp or by the constituent amino acids Trp and Ala (Fig. 5C). Similar results were obtained using Leu-Ala and Arg-Ala (data not shown). These results indicated that the relevant signaling molecule in this process is a dipeptide bearing a destabilizing N-terminal residue. The range of dipeptide concentrations that significantly stimulated Cup9p degradation was similar to physiologically active levels of many other nutrients.

Cup9p represses transcription of the transporter-encoding *PTR2* gene (27). Thus, the dipeptide-induced acceleration of Cup9p degradation would be expected to increase the levels of *PTR2* mRNA, ultimately leading to an increase in dipeptide uptake. This conjecture was tested by examining the levels of *PTR2* mRNA in the presence or absence of dipeptides in the medium. At 25 μM, both Trp-Ala and Arg-Ala induced *PTR2* expression in

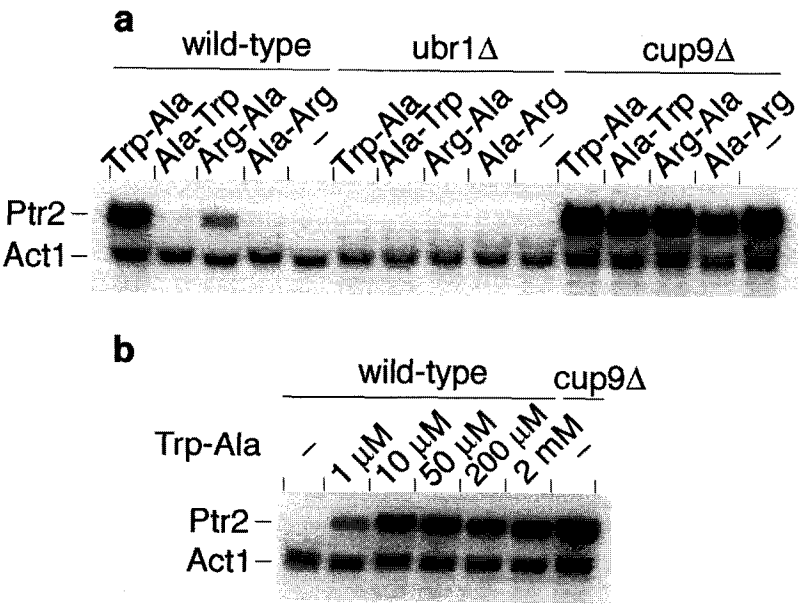

Figure 6. Effects of dipeptides on expression of the dipeptide transporter gene *PTR2*. (**A**) Induction of *PTR2* expression by dipeptides bearing destabilizing N-terminal residues (Trp-Ala and Arg-Ala) required both *UBR1* and *CUP9*. Dipeptides bearing a stabilizing N-terminal residue (Ala-Trp and Ala-Arg) had no effect on *PTR2* expression. *PTR2* mRNA and the *ACT1* mRNA loading control are indicated. (**B**) Effect of different concentrations of Trp-Ala on the levels of *PTR2* mRNA (2).

the wildtype (*UBR1*) strain (Fig. 6A). Both Ubr1p and Cup9p were required for these effects, since the expression of *PTR2* was not altered by dipeptides in *ubr1Δ* and *cup9Δ* strains. Testing a range of concentrations of Trp-Ala showed that induction of *PTR2* mRNA could be observed at 1 μM Trp-Ala (Fig. 6B), increasing at higher dipeptide concentrations, in agreement with the observed changes in the half-life of Cup9p (Fig. 5C).

A plausible mechanism of the enhancement effect is that a dipeptide interacts with either the basic or hydrophobic N-terminal residue-binding sites of Ubr1p, while a distinct (third) substrate-binding site of Ubr1p

recognizes the internal degron of Cup9p. In this model, the interaction of Ubr1p with dipeptides allosterically increases the ability of the Ubr1p-Rad6p (E3-E2) complex to ubiquitylate Cup9p. To test whether dipeptides act directly through Ubr1p, we examined the effect of dipeptides on Cup9p ubiquitylation in an *in vitro* system consisting of the following purified components: Ubr1p (E3), Rad6p (E2), Uba1p (E1), Ub, ATP, and radiolabeled Cup9p. In this system, Cup9p was significantly multi-ubiquitylated, in a Ubr1p/Rad6p-dependent reaction (data not shown), in the absence of added dipeptides (2). This result was consistent with the relatively rapid *in vivo* degradation of Cup9p ($t_{1/2}$ ~5 min) in the absence of dipeptides (Fig. 5C). The addition of dipeptides bearing either type of destabilizing N-terminal residue to the *in vitro* system substantially stimulated the Ubr1p-dependent multi-ubiquitylation of Cup9p. Dipeptides of the same composition but bearing a stabilizing N-terminal residue did not stimulate multi-ubiquitylation, nor did the amino acid components of these dipeptides (2). These results demonstrated that dipeptides act directly through Ubr1p, without an intermediate signaling pathway. The underlying allosteric mechanism may involve increased affinity of Ubr1p for Cup9p, or enhanced ubiquitylation activity of the Ubr1p-Rad6p complex towards Cup9, or both.

Thus, the two binding sites of Ubr1p that interact with destabilizing N-terminal residues can act as allosteric sites that enable Ubr1p to sense the presence of imported dipeptides, and to accelerate degradation of the Cup9p repressor, resulting in an appropriate induction of the Ptr2p transporter (Fig. 7). This model predicts that a dipeptide bearing a destabilizing N-terminal residue, e. g., Leu-Ala, should stimulate its own uptake, in contrast to Ala-Leu. This prediction was borne out when we tested the ability of these two leucine-containing dipeptides to support the growth of *S. cerevisiae* auxotrophic for leucine (2).

This work (2) established for the first time that the activity of an E3 can be directly linked to the presence of an environmental signal through an allosteric interaction with a small compound. Specifically, dipeptides bearing destabilizing N-terminal residues are shown to act as allosteric activators of Ubr1p, enhancing its ability to support the ubiquitylation and degradation of Cup9p. Physiologically, this results in a positive feedback circuit governing the uptake of peptides (Fig. 7). Through their binding to

Ubr1p, the imported dipeptides accelerate degradation of Cup9p, thereby derepressing the synthesis of the Ptr2p transporter and enhancing the cell's

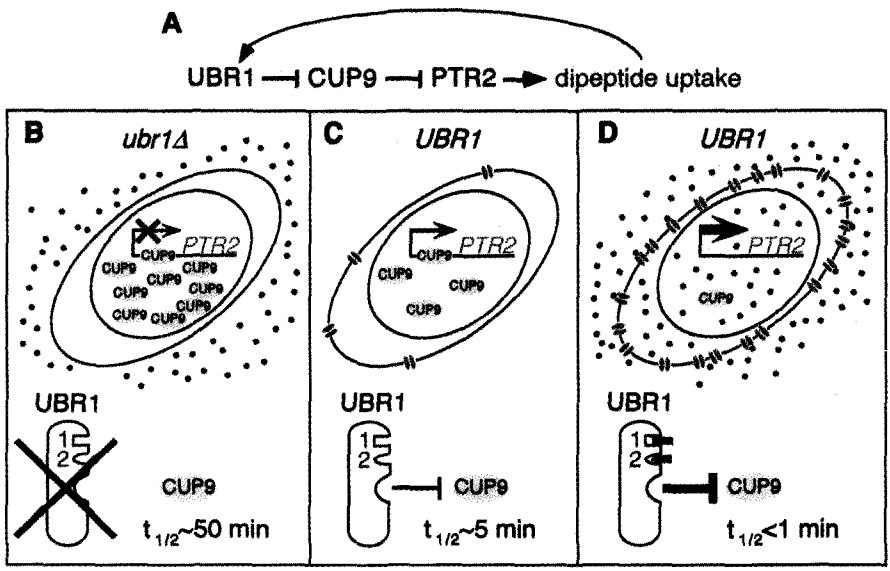

Figure 7. Feedback regulation of peptide import in *S. cerevisiae*. (**A**) Genetic diagram of the peptide transport circuit. (**B**) UBR1 is required for dipeptide uptake. In the absence of Ubr1p (*ubr1Δ*), the transcriptional repressor CUP9 is long-lived, accumulates to high levels, and extinguishes the expression of the *PTR2* gene. Thus, *ubr1Δ* cells cannot import dipeptides (red dots). (**C**) In a wildtype (*UBR1*) cell growing in the absence of extracellular dipeptides, UBR1 targets CUP9 for degradation ($t_{1/2}$ ~5 min), resulting in a moderate concentration of CUP9 and weak but significant expression of the PTR2 transporter (blue double ovals). (**D**) In wildtype (*UBR1*) cells growing in the presence of extracellular dipeptides some of which bear destabilizing N-terminal residues, the imported dipeptides bind to either the basic (type 1) or the hydrophobic (type 2) residue-binding site of UBR1. These peptides are denoted as a red block and a green wedge, respectively. Binding of either type of dipeptide to UBR1 allosterically increases the rate of UBR1-mediated degradation of CUP9. Peptides of both types are shown as bound to UBR1, but in fact the binding of either peptide accelerates CUP9 degradation. The resulting decrease of the half-life of CUP9 from ~5 min to less than 1 min results in a very low concentration of CUP9, and consequently to a strong induction of the PTR2 transporter (2).

ability to import di- and tripeptides. As most cells have the capacity to import peptides, the results of this work suggest that peptide import may be regulated similarly by Ubr1p homologs in metazoans and by the ClpAP-dependent N-end rule pathway in *E. coli*. The Ub system is either known or suspected to play major roles in the control of intermediary metabolism and the transport of small molecules across membranes. The above findings (2) suggest that these compounds, or their enzymatically produced derivatives, may modulate the functions of E3s in the Ub system similarly to the effects observed with dipeptides and Ubr1p.

The N-End Rule Pathway and Fidelity of Chromosome Segregation

Cohesion between sister chromatids is established during DNA replication and depends on a protein complex called cohesin (37-39). Previous work by Uhlmann et al. (40) has shown that the metaphase-anaphase transition in *S. cerevisiae*, the *ESP1*-encoded protease, termed separase, cleaves Scc1p, a 63K subunit of cohesin. The resulting 33K C-terminal fragment of Scc1p bears N-terminal arginine, a destabilizing residue in the N-end rule (19). More recently, it was demonstrated that the Scc1p fragment is indeed short-lived *in vivo*, and that is destroyed by the N-end rule pathway (3). Moreover, overexpression of a long-lived derivative of the Scc1p fragment was found to be lethal. These results led Rao et al. to examine chromosome stability in *ubr1*Δ cells, which lack the N-end rule pathway, revealing a strikingly increased frequency of chromosome loss (Fig. 8). The bulk of increased chromosome loss in *ubr1*Δ cells was shown to be caused by metabolic stabilization of the ESP1-produced SCC1 fragment (3). This fragment is the first physiological substrate of the N-end rule pathway that is targeted through a substrate's N-terminal residue. A number of yeast proteins bear putative cleavage sites for the ESP1 separin, suggesting other physiological substrates and other functions of the N-end rule pathway (3).

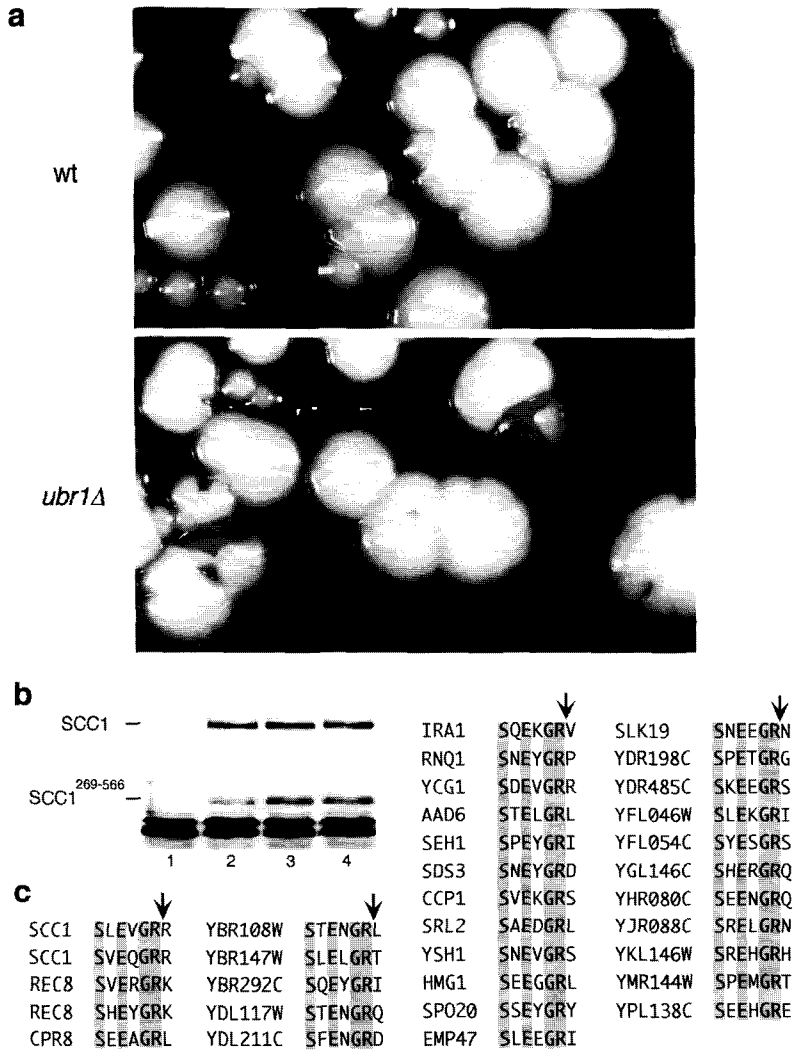

Figure 8. Increased chromosome instability in *ubr1*Δ *S. cerevisiae* (3). (**a**) Sectoring assay for the chromosome loss using the *SUP11*-marked YPH277 (*UBR1*) strain and its *ubr1*Δ derivative (see Methods). (**b**) Immunoblot analysis of Arg-Scc1p$^{269\text{-}566h}$ and Gly-Scc1p$^{269\text{-}566h}$, produced through Esp1p-mediated cleavage of normally expressed, C-terminally ha-tagged Scc1p in wild-type and *ubr1*Δ strains. Yeast cultures (OD_{600} of ~0.4) were processed for immunoblotting with

anti-ha antibody. Lane 1, YPH277 (control) cells lacking the ha-tagged Scc1p. Lane 2, AVY129 (*UBR1*) cells expressing normal levels of $Scc1p^{R180E}$-ha. Lane 3, the same as in lane 2 but with AVY132 (*ubr1*Δ) cells. Lane 4, the same as in lane 2, but with $Scc1p^{R180E,\ R269G}$-ha, bearing the Arg→Gly mutation at position 269. The bands of full-length Scc1p-ha and its Esp1p-produced $SCC1^{269\text{-}566h}$ fragments are indicated on the left. (**c**) *S. cerevisiae* proteins containing putative cleavage sites for the Esp1p separase. The query SxExGRx and the Pattern Match program (http://genome-www2.stanford.edu/cgi-bin/SGD/PATMATCH/nph-patmatch) were used to search the *S. cerevisiae* genome database (SGD). Arrows indicate the inferred sites of cleavage by Esp1p.

Concluding Remarks

The Ub system is a set of interacting but quasi-independent, similarly organized Ub-conjugating pathways that converge on the 26S proteasome, delivering to it for degradation a vast range of substrates bearing covalently linked multi-Ub chains. There are dozens of Ub-conjugating pathways in yeast and possibly hundreds in larger eukaryotes. Distinct Ub-conjugating pathways differ, among other things, in the composition of their E2-E3 targeting complexes, which determine the range of degrons recognized by a specific pathway. These pathways are alike in their dependence on the Ub conjugation and the proteasome, hence the recurring mechanistic similarities in the pathways' designs. This brief description does not encompass the entire Ub-proteasome system, because some proteins are degraded by the 26S proteasome in a Ub-independent manner, and some Ub-conjugating pathways target proteins either for degradation in the lysosome (as distinguished from the proteasome) or for the fates other than degradation.

The N-end rule pathway is one of many Ub-dependent proteolytic pathways. Having been the first such pathway defined through molecular genetic methods (18, 19), the N-end rule pathway was the setting where several findings relevant to the entire Ub system were first made, the discovery of multi-Ub chains being one of them (12). The emerging functions of this pathway in mammals are described elsewhere (44, 45).

Acknowledgments

I am most grateful to the graduate students and postdoctoral fellows, both former and current, whose research in my laboratory over the last two decades made possible all of the lab's contributions to the Ub field. Three recent studies described above were carried out by Dr. Youming Xie, Dr. Glenn Turner, Dr. Hai Rao, and Fangyong Du, a graduate student. Dr. Rao's work on the Scc1p-Esp1p-Ubr1p circuit was a collaboration with Dr. F. Uhlmann (ICRF, London, U.K.) and Dr. K. Nasmyth (IMP, Vienna, Austria). Work in my laboratory is supported by grants from the National Institutes of Health and the Kirsch Foundation.

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Phosphorylation-Dependent Substrate Recognition in Ubiquitin-Mediated Proteolysis

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Abstract

The selection of protein substrates for ubiquitination is commonly achieved by the specific interactions of E3 protein-ubiquitin ligases with both target proteins and E2 enzymes. The protein-protein interactions that confer selectivity to the ubiquitination machinery have many features in common with the modular protein interactions characteristic of signal transduction pathways. Notably, recruitment of a target protein into an E3 protein-ubiquitin ligase complex is often dependent on its phosphorylation on either serine/threonine or tyrosine residues. We discuss examples of phospho-dependent protein ubiquitination in signal transduction and the cell cycle, and suggest that viral proteins, such as the Epstein-Barr virus polypeptide LMP2A, can stimulate the ubiquitination of host cell proteins by serving as a scaffold to recruit protein-ubiquitin ligases and their substrates. We summarize recent data suggesting that multi-site phosphorylation of the yeast CDK inhibitor Sic1 is required for its binding to an E3 complex, and

consequent destruction. Based on experimental observation and theoretical modeling, we argue that this requirement for multi-site phosphorylation of Sic1 during the G1 phase of the cell cycle yields a switch-like effect which is important for regulating entry into S phase.

Modular Protein Interaction Domains Regulate Dynamic Cellular Behaviour

The behaviour of eukaryotic cells is controlled by their responses to external signals and internal cues. Commonly, the biochemical pathways that couple the receptors which sense signal inputs to their ultimate intracellular targets are composed of proteins that contain multiple, independently folding protein modules (Pawson, 1995). These protein domains either mediate specific protein-protein, protein-phospholipid or protein-nucleic acid interactions, or catalyze reactions such as phosphorylation (Pawson and Scott, 1997). Interaction domains play a central role in signal transduction, and indeed in virtually all aspects of dynamic cellular regulation, by directing proteins to their upstream activators and downstream targets, and thereby ensuring specificity in the cellular response to activated receptors (Pawson and Nash, 2000). Importantly, post-translational modifications such as phosphorylation often exert their effects by creating binding sites for the interaction domains of target proteins, which are then regulated through their recruitment into specific signaling complexes.

This scheme is typified by the binding of Src homology 2 (SH2) domains (Sadowski et al., 1986) of cytoplasmic proteins to phosphotyrosine sites on activated growth factor receptors (Figure 1). SH2 domains are a common feature of an otherwise biochemically diverse group of proteins that mediate the biological functions of receptor protein-tyrosine kinases (Pawson, 1995; Pawson and Scott, 1997). SH2 domains recognize short peptide motifs in a fashion that is dependent on phosphorylation of the binding site on tyrosine, and also on the nature of the amino acids immediately C-terminal to the phosphotyrosine (Anderson et al., 1990; Moran et al., 1990; Songyang et al., 1993). Thus the autophosphorylation of activated receptor tyrosine kinases creates specific binding sites for the SH2 domains of intracellular targets, which following their recruitment to the

receptor regulate events such as phospholipid metabolism, activation of Ras-like GTPases, gene expression, cytoskeletal architecture, and protein trafficking and stability. Extensive structural and functional analysis has revealed the mechanism through which phosphotyrosine recognition and specificity is achieved, and has underscored the importance of these events in normal mammalian development, in the development of cancer cells, and in inherited disorders (Kimber et al., 2000; Kuriyan and Cowburn, 1997; Li et al., 1999; Pawson and Nash, 2000; Puil et al., 1994; Saxton et al., 2001).

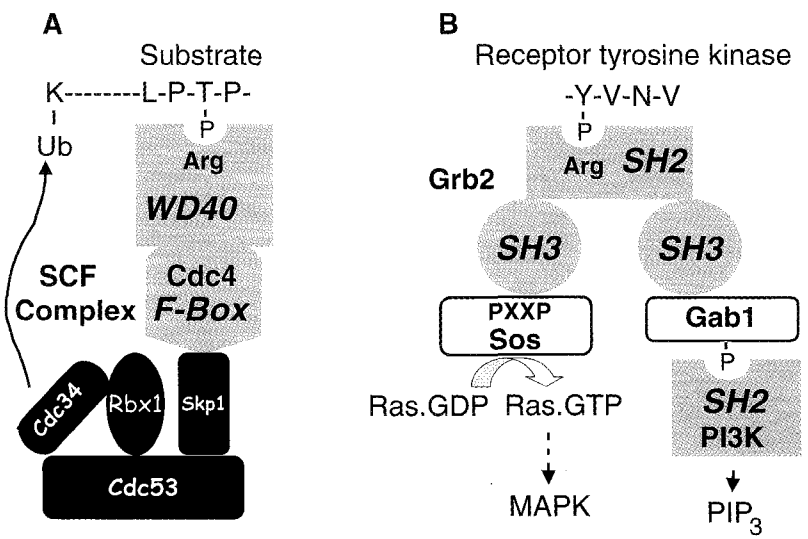

Figure 1. Modular phospho-dependent protein-protein interactions are employed in receptor tyrosine kinase signaling to the Ras-MAP kinase pathway, and also in the ubiquitination of target proteins by SCF complexes. The WD40 repeat domain of an F-box protein such as Cdc4 binds a phosphothreonine-containing motif on the substrate for ubiquitination, which is thereby recruited to the SCF E3 complex (A), while the SH2 and SH3 domains of Grb2 link phosphotyrosine sites on activated receptor tyrosine kinases to targets such as Sos, that regulate downstream signaling pathways (B). In both cases, recognition of the phosphorylated motifs appears to involve conserved arginine residues.

SH2 domains exemplify a number of characteristics of protein interaction domains. They are modular, in the sense that they fold independently and retain their binding properties when expressed in isolation. Their N- and C-termini are typically close to one another, and on the opposite face from the ligand-binding site, a feature that has likely facilitated their insertion into host proteins during evolution. In addition, they recognize short defined peptide sequences within their binding partners. Furthermore, they are found in a large group of cytoplasmic proteins, suggesting that the re-iterated use of the simple SH2 domain-phosphotyrosine recognition device is used to link tyrosine kinase signaling to a broad range of intracellular effectors. Specific interaction domains are frequently found in tens or hundreds of different proteins, consistent with the view that they provide an organizational framework for the assembly of signaling networks within the cell.

Recent data have indicated that protein phosphorylation has a more general role in controlling protein-protein interactions. For example, the PTB domains of signaling proteins such as Shc and IRS-1 also bind phosphotyrosine-containing motifs, such as those found on the insulin- and nerve growth factor receptors, but do so in a quite different manner to SH2 domains (Forman-Kay and Pawson, 1999). In addition, a growing number of proteins and interaction domains have been shown to recognize phosphoserine- and phosphothreonine-containing sequences, suggesting that protein-serine/threonine kinases, as well as protein-tyrosine kinases, modify cellular function through their ability to regulate protein-protein interactions. Phosphoserine/threonine recognition was first discovered as a property of 14-3-3 proteins, and has subsequently been identified for FHA domains, as well as WD40 repeat domains (see below) and a subset of WW domains (Yaffe and Elia, 2001). These phospho-dependent interactions can regulate protein function either by localizing polypeptides to a specific site in the cell, by altering their enzymatic activity, or by controling the association of enzymes with their substrates, and thereby assuring specificity in processes such as signal transduction or the cell cycle (Pawson et al., 2001).

The binding of interaction domains to their targets is not always dependent on post-translational modifications. Notably, a number of modules, including SH3, WW and EVH1 domains, recognize short proline-rich motifs that adopt a polyproline type II helix, and these interactions

generally do not require phosphorylation (Pawson and Nash, 2000). Interestingly, it is possible to build up quite complex networks of protein-protein interactions through the combinatorial use of distinct types of interaction domains. As a simple example, the adaptor protein Grb2 is composed of a single SH2 domain, flanked by two SH3 domains, and therefore physically couples activated receptor tyrosine kinases to cytoplasmic targets with proline-rich sequences such as Sos, a guanine nucleotide exchange factor for the Ras GTPase, and Gab1, a scaffolding protein that is itself a substrate for tyrosine phosphorylation and consequent binding of SH2 proteins such as phosphatidylinositol (PI) 3'-kinase (Pawson, 1995; Pawson and Nash, 2000; Pawson and Scott, 1997). Thus Grb2 links specific phosphotyrosine (pTyr-X-Asn) motifs to both the Ras-MAP kinase pathway, and pathways controlled by PI-3,4,5-P_3 (Figure 1).

Protein-Protein Interactions Determine Specificity in Ubiquitin-Mediated Proteolysis

The types of modular protein-protein interactions originally identified in signaling pathways from cell surface receptors also play a central role in selectively targeting proteins for ubiquitination and degradation (Figures 1 and 2). The E3 components of the ubiquitination cascade, which direct the final step in protein ubiquitination, physically recruit their targets into a multi-component complex that also contains an E2 enzyme. E3s therefore serve to physically link the ubiquitination machinery to a specific protein substrate. This is evident both for E3 enzymes that carry their own protein-ubiquitin ligase (Hect) domain (Huang et al., 1999; Huibregtse et al., 1995), or E3 proteins (or complexes) that lack intrinsic enzymatic activity, but recruit both the substrate and an E2 protein-ubiquitin ligase (i.e. through a RING-H2 finger domain) (Zheng et al., 2000). The N-terminal region of c-Cbl, for example, contains a variant SH2 domain which binds phosphotyrosine sites on activated receptor tyrosine kinases, linked to a central RING-H2 finger domain that associates with E2 protein-ubiquitin ligases (Meng et al., 1999; Waterman et al., 1999). Thus c-Cbl serves as an adaptor that recruits an E2 enzyme to activated receptors, promoting their ubiquitination and downregulation (Joazeiro et al., 1999).

SCF multi-protein complexes, described in detail below, perform a somewhat similar function, in that the target for ubiquitination binds the C-terminal interaction domain of an F-box protein, and is thereby juxtaposed to an E2 enzyme (Patton et al., 1998). In several cases, the binding of the ubiquitination target to the C-terminal region of the F-box protein, often composed of WD40 repeats, is dependent on phosphorylation of the substrate on serine/threonine (Figure 1). Examples include the phospho-dependent ubiquitination and degradation of cell cycle regulators such as yeast Sic1 and human cyclin E (see below), and of IκB, the inhibitory subunit of the NFκB transcription factor (Karin and Ben-Neriah, 2000; Koepp et al., 2001; Moberg et al., 2001; Nash et al., 2001; Strohmaier et al., 2001). In this latter case, signaling from TNF receptors activates an IKK kinase, which phosphorylates IκB on two closely spaced serine residues; the phosphorylated IκB is then recognized by the F-box protein βTrCP, and consequently recruited into an SCF E3 complex and ubiquitinated (Karin and Ben-Neriah, 2000; Maniatis, 1999). Destruction of IκB liberates an active NFκB transcription factor.

Similar to the RING-H2/SCF E3 complexes, the Hect family of E3 protein-ubiquitin ligases bind their targets through a non-catalytic interaction sequence, providing specificity in substrate recognition.(Figure 2). The Nedd4 family of Hect domain proteins (Harvey and Kumar, 1999) contain an N-terminal C2 domain, involved in apical membrane localization, followed by 3-4 WW domains, which typically recognize PPXY motifs (Staub et al., 2000). Targets for ubiquitination by Nedd4, such as the epithelial sodium channel (ENaC), are recruited through binding of their PPXY motifs to the Nedd4 WW domains, and are therefore positioned to accept ubiquitin from the C-terminal Hect catalytic domain. Interestingly, mutations in the WW domain-binding sites of ENaC cause an inherited hypertensive disorder, Liddle's syndrome. Another example is provided by the binding of Nedd4 family members to the Epstein-Barr virus (EBV) latent membrane protein (LMP) 2A, a polypeptide with multiple membrane-spanning elements which is expressed in latently EBV-infected B cells (Longnecker and Miller, 1996). One function of LMP2A is apparently to block signaling from the B cell antigen receptor (BCR), a process that is normally dependent on the cytoplasmic tyrosine kinases Lyn and Syk (Cheng and Chan, 1997; Cheng et al., 1995). BCR signaling involves the

phosphorylation of the Igα and Igβ receptor subunits by a Src family kinase such as Lyn, which in turn creates docking sites for the tandem SH2 domains of Syk. The N-terminal cytoplasmic region of LMP2A has several binding motifs for the interaction domains of B cell signaling proteins, and is modified by tyrosine phosphorylation in infected B cells. In particular, phosphorylated LMP2A has binding sites for the SH2 domains of Lyn and Syk, which are therefore recruited into a complex with the viral protein (Longnecker and Miller, 1996). In addition, LMP2A proteins have two conserved PPPPY motifs, which we and others have found bind the WW domains of the E3 protein-ubiquitin ligase AIP4, and other members of the Nedd4 family (Figure 3) (Ikeda et al., 2000; Winberg et al., 2000).

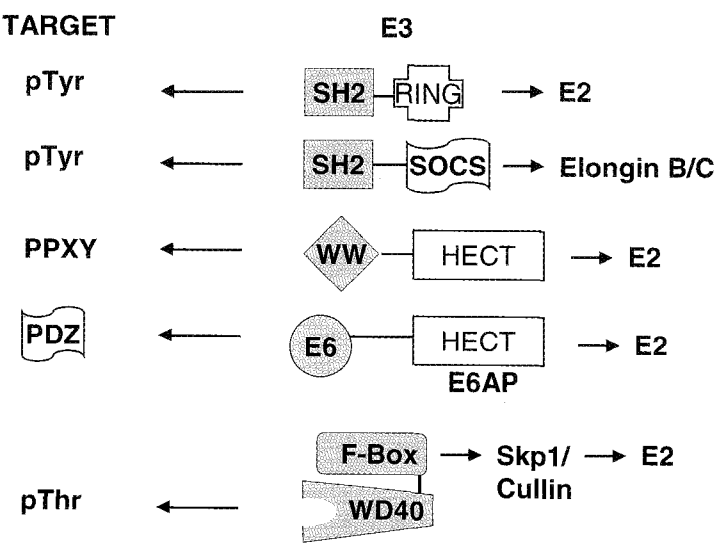

Figure 2. Protein interaction domains are used by E3 protein-ubiquitin ligases to recruit substrates for ubiquitination, as well as E2 enzymes. Ubiquitination targets contain specific peptide motifs which bind interaction domains in the E3 protein/complex. These interactions can require phosphorylation on tyrosine (pTyr) or threonine (pThr), or the presence of proline-rich motifs (PPXY). The papilloma virus E6/E6AP complex can recognize substrates that contain PDZ domains, which bind a C-terminal motif in the E6 viral protein. See text for details.

Recruitment of AIP4 into the LMP2A complex promotes the ubiquitination and destabilization of Lyn, and potentially other proteins, and this may contribute to the effect of LMP2A on EBV-infected B cells, and maintenance of the latent state (Winberg et al., 2000). LMP2A exemplifies the ability of pathogenic micro-organisms to usurp the modular protein-protein interactions that normally control cellular function, and thereby to rewire cellular behaviour to suit the needs of the virus or bacterium (i.e. Gruenheid et al., 2001).

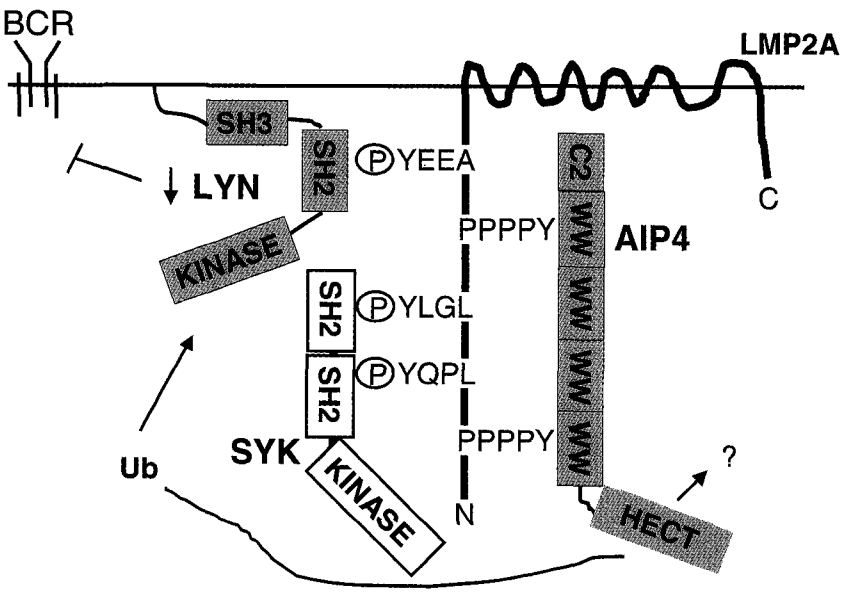

Figure 3. The EBV protein LMP2A binds the SH2 domains of the Lyn and Syk B cell tyrosine kinases, and the WW domains of the AIP4 E3 protein-ubiquitin ligase. The protein-protein interactions elicited by the viral protein lead to the ubiquitination and destabilization of Lyn (and potentially other B cell proteins), which may contribute to the inhibition of BCR signaling in latently infected cells.

These examples illustrate the importance of protein-protein interactions in recruiting protein-ubiquitin ligases and their substrates into common complexes that ensure the specificity of substrate recognition. It is evident

that phosphorylation of the target often controls recognition by the substrate-binding domain of the E3, but rather little is known concerning the precise mechanisms through which serine/threonine phosphorylation regulates protein ubiquitination. To begin to address this issue we expand our discussion of SCF complexes, the family of multi-protein ubiquitin ligases that recruit targets via substrate-specific adapter subunits called F-box proteins (Patton et al., 1998). We explore the mechanism whereby the yeast F-box protein Cdc4 recognizes one of its substrates, the Cdk inhibitor Sic1, and discuss the importance of this phospho-dependent interaction for control of the eukaryotic cell cycle.

Cdk Activity and the Control of DNA Replication

In the eukaryotic cell cycle, the once and only once per cell cycle replication of the genome is dictated primarily by the periodic appearance and disappearance of cyclin dependent kinase (Cdk) activity (Nasmyth, 1996). In G1 phase, when Cdk activity is low, multi-protein complexes assemble on origins of DNA replication thereby rendering origins competent to initiate replication. Once Cdk activity appears at the beginning of S-phase, loaded replication origins fire in response to multiple phosphorylation events catalyzed by Cdks and downstream kinases. Importantly, in the continued presence of Cdk activity, origins are prevented from re-loading, thereby ensuring that replication is initiated only once. After cells have completed mitosis, Cdk activity is turned off as cells re-enter G1 phase, where origins can once again be assembled. The mechanisms underlying cell cycle regulated periodicity of Cdk activity have been elucidated primarily in yeast, but appear to operate in a similar manner in metazoans. In general terms, Cdk activity is turned on in late G1 phase through transcriptional induction of cyclin gene expression and degradation of Cdk inhibitors, whereas at the end of mitosis, Cdk activity is shut down through degradation of mitotic cyclins. Multi-subunit E3 enzyme complexes termed the SCF (for Skp1-Cdc53/cullin-F-box protein) and the APC (for Anaphase Promoting Complex/Cyclosome) mediate degradation of cell cycle proteins at the G1 to S and M to G1 transitions respectively (see Figure 4).

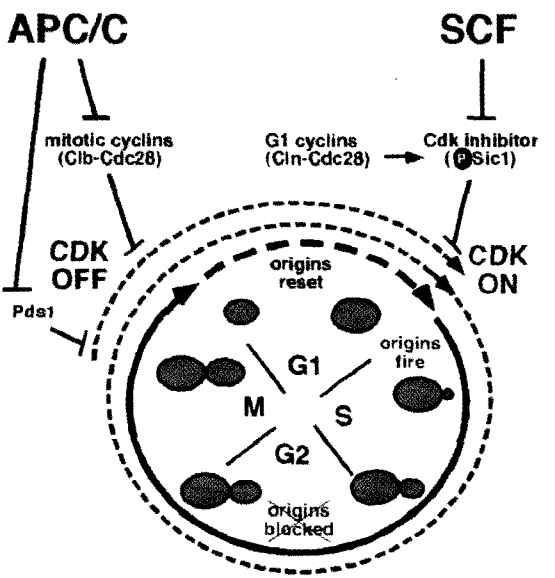

Figure 4. Ubiquitin-mediated proteolysis regulates the onset and demise of Cdk activity during the cell division cycle. The Anaphase Promoting Complex/Cyclosome (APC/C) is active from the onset of anaphase until the end of G1 phase, during which it targets mitotic cyclins (Clbs) and other proteins such as Pds1. The SCF^{Cdc4} complex is constitutively active but only targets Sic1 and other substrates once they have been specifically phosphorylated by G1 cyclin (Cln)-Cdk (Cdc28) activity. See text for details.

In yeast, a number of critical cell cycle events including DNA replication, bud emergence, and spindle pole body duplication are coordinated at Start, the point of commitment to a new round of cell division that is analogous to the Restriction Point in mammalian cells (Cross, 1995). The Cdk inhibitor Sic1 plays a critical role in determining the onset of DNA replication because it holds B-type cyclin (Clb)-Cdc28/Cdk1 activity in check until appropriate signals dictate a new round of cell division (Mendenhall, 1993; Nugroho and Mendenhall, 1994). In cells that lack *SIC1*, replication is uncoupled from other events at Start such that it occurs almost immediately after cells have completed the preceding mitosis (Nugroho and Mendenhall, 1994; Schwob et al., 1994). The inhibition of

Clb5/6-Cdc28 activity by Sic1 is crucial for maintaining the Cdk-free window in G1 phase needed for proper assembly of replication origins. As a consequence of incomplete origin assembly and replication, massive genome instability occurs in *sic1* strains, particularly in daughter cells, which have a larger growth requirement and hence a longer G1 phase than mother cells (Nugroho and Mendenhall, 1994). Sic1 also plays an important role in re-establishing G1 phase at the end of mitosis, where it conspires with the APC/C activator Cdh1 to fully shut down Cdk activity, as illustrated by the inviability of *sic1 cdh1* double mutants (Calzada et al., 2001; Schwab et al., 1997; Visintin et al., 1997). In summary, the timely elimination of Sic1 is crucial for proper replication control and attendant genome stability.

Discovery of SCF Ubiquitin Ligases

SCF complexes were first discovered via the striking multi-budded, G1 phase arrest phenotype caused by loss of SCF function, as occurs when *cdc4*, *cdc34* and *cdc53* mutants are shifted to the non-permissive temperature (Patton et al., 1998). The first genetic evidence that this arrest phenotype might arise from perturbation of a ubiquitin-mediated proteolytic pathway was the identification of Cdc34 as an E2 enzyme (Goebl et al., 1988). The critical G1 phase target of Cdc34 is Sic1, as shown by the elegant genetic result in which the *cdc34* phenotype is bypassed by disruption of *SIC1* (Schwob et al., 1994). Synthetic lethal and physical interactions amongst *CDC4/34/53* and their gene products suggested a common function for these genes (Mathias et al., 1996; Willems et al., 1996). High copy suppression analysis of the *cdc4-1* mutation and a hunt for interaction partners of Cdc4 identified another pathway component, the adapter protein Skp1 (Bai et al., 1996). Alignment of Skp1-binding proteins revealed a motif termed the F-box, which corresponds to the Skp1 interaction surface (Bai et al., 1996; Patton et al., 1998; Schulman et al., 2000). Biochemical purification of Cdc53/cullin and Skp1 complexes from yeast and mammalian cells uncovered a final core component, variously called Rbx1, Hrt1 or Roc1, a small protein containing a RING-H2 finger domain (Kamura et al., 1999; Seol et al., 1999; Skowyra et al., 1999). Other

factors associated with SCF complexes include the ubiquitin-like modifier Nedd8 (Lammer et al., 1998) and another Skp1-binding protein called Sgt1 (Kitagawa et al., 1999).

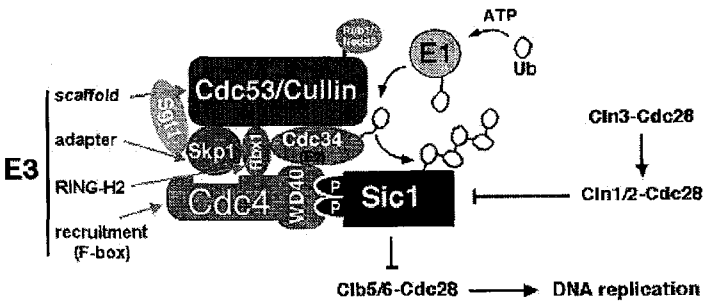

Figure 5. Architecture of SCF complexes. A core complex composed of Skp1, Cdc53/Cullin and Rbx1/Roc1/Hrt1 couples to substrate specific adapter subunits called F-box proteins. As shown, the SCF^{Cdc4} complex recognizes phosphorylated forms of Sic1 only once it has been phosphorylated by the Cln1/2-Cdc28 kinases. Cln3 is an upstream G1 cyclin that is specialized for activation of G1-specific transcription, including the *CLN1/2* genes. In the absence of Sic1 degradation, the Clb5/6-Cdc28 complexes cannot be activated, resulting a failure to initiate DNA replication and consequent G1 phase arrest.

Biochemical analysis of the Sic1 degradation machinery revealed that the components genetically implicated in Sic1 degradation form a multi-protein E3 complex that specifically recognizes and ubiquitinates phosphorylated forms of Sic1 (Feldman et al., 1997; Skowyra et al., 1997). SCF complexes are based on a core machinery, composed of Skp1, Cdc53/Cul1 and Rbx1, which recruits substrates via any one of a number of F-box protein adapter subunits. Cdc53 serves as a scaffold protein that links the Skp1-F-box protein subcomplex to the RING-H2 protein Rbx1 and, usually, the E2 enzyme Cdc34. F-box proteins have a bipartite structure: the N-terminal F-box motif binds tightly to Skp1, whereas C-terminal protein-protein interaction domains bind to substrates. Typically, F-box proteins contain WD40 or leucine rich repeat (LRR) interaction domains. Most often, only the phosphorylated forms of substrates are recognized by F-box proteins, thereby linking kinase-based signaling networks to the ubiquitin system.

Rbx1, together with a distant homolog, the APC subunit Apc11, were the first defining members of what is now the largest class of E3 enzymes, the RING-H2 family (Tyers and Jorgensen, 2000). Other cullin based complexes, including the VHL-Elongin BC-Cul2 complex that uses a different class of adapter subunits called SOCS box proteins, also require Rbx1 for activity (Kamura et al., 1999). The RING-H2 domain binds and may also activate E2 enzymes (Seol et al., 1999; Zheng et al., 2000). The overall architecture for SCF complexes shown in Figure 5.

Phosphorylation-Dependent Substrate Recognition by SCF Complexes

Sic1 is targeted for degradation upon its phosphorylation by the G1 form of Cdk activity, the Cln-Cdc28 kinases, which are immune to Sic1 inhibition (Verma et al., 1997). Under appropriate growth conditions, Cln-Cdc28 activity is induced towards the end of G1 phase as cells prepare to commit to a new round of division (Tyers et al., 1993). Genetic analysis has shown that the critical target of Cln-Cdc28 activity is Sic1 (Schneider et al., 1996; Tyers, 1996). The primary sequence of Sic1 contains nine Cdk consensus phosphorylation sites, S/T-P-XK/R (Figure 6). Mutation of 2 or 3 Cdk sites is sufficient to stabilize Sic1 in vivo and cause G1 arrest, at least under conditions when the mutant protein is over-expressed (Verma et al., 1997). The cognate F-box protein for Sic1 is Cdc4, which harbors a C-terminal WD40 repeat domain. The Sic1 degradation pathway has been completely reconstituted from recombinant proteins (Feldman et al., 1997; Skowyra et al., 1997). First, phosphorylation of Sic1 by purified Cln2-Cdc28 kinase drives its high affinity interaction with Cdc4. Once recruited to SCF^{Cdc4}, Sic1 is efficiently ubiquitinated in the presence of the E2 enzyme Cdc34, E1, ubiquitin and ATP. Importantly, Cdc4 is highly selective for phospho-Sic1 as compared to other F-box proteins (Skowyra et al., 1997). Finally, in vitro ubiquitinated Sic1 can be recognized and degraded by purified proteasome preparations (Verma et al., 2001).

In addition to Sic1, Cdc4 targets a number of other important substrates including the replication factor Cdc6, the transcription factor Gcn4 and the

polarization effector/Cdk inhibitor Far1 (Tyers and Jorgensen, 2000). In each case, substrate degradation is triggered upon Cdk-dependent phosphorylation, usually on multiple sites. At least one other F-box protein in yeast targets its substrates in a phosphorylation dependent manner. The F-box protein Grr1, which contains an LRR domain, targets the G1 cyclins Cln1 and Cln2 (Barral et al., 1995; Skowyra et al., 1999). As for Sic1, mutation of several Cdk phosphorylation sites in Cln2 stabilizes the protein in vivo (Lanker et al., 1996). SCF-dependent ubiquitination of Cln1/2, Cdc6 and Gcn4 has been accomplished with purified recombinant proteins (Chi et al., 2001; Elsasser et al., 1999; Skowyra et al., 1999).

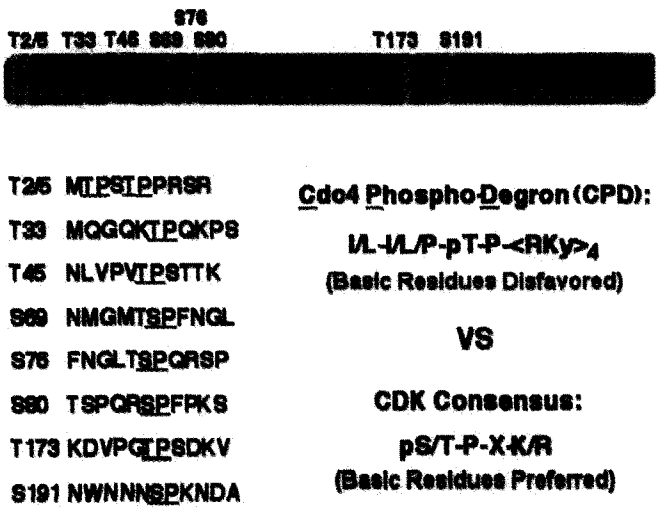

Figure 6. Multi-site phosphorylation of Sic1 is required for recognition by Cdc4. The Cdk consensus phosphorylation site sequence is contrasted with the Cdc4 Phospho-Degron (CPD) consensus sequence. < > indicates disfavoured residues in the CPD motif.

Conservation of SCF Function in the Cell Cycle

As anticipated following their discovery in yeast, SCF complexes also play a key role in the mammalian cell cycle. The LRR containing F-box protein Skp2 recognizes phosphorylated forms of the Cdk inhibitor $p27^{Kip1}$, which normally restrains entry into S-phase by inhibiting the G1 cyclin-Cdk activity, primarily cyclin E-Cdk2 (Amati and Vlach, 1999). Growth factor treatment causes a number of events that culminate in p27 destruction, including induction of D-type cyclins, which sequester p27 away from cyclinE-Cdk2, increased expression of cyclin E itself and increased expression of Skp2. Importantly, cyclin E-Cdk2 directly phosphorylates p27 thereby driving its capture by Skp2 and subsequent degradation (Carrano et al., 1999). Thus, the p27-cyclin E couple is bistable, existing in a state of high p27 and low cyclin E or a state of low p27 and high cyclin E. Once cyclinE-Cdk2 activity is liberated, the key events of centrosome duplication and DNA replication are set in motion (Winston et al., 1999). The importance of proper p27-cyclinE regulation is illustrated on the one hand by the fact that p27 is a haploinsufficient tumour suppressor (Fero et al., 1998), and on the other by the observation that deregulated cyclin E activity leads to genome instability in tissue culture cells (Spruck et al., 2001). Phosphorylation-dependent ubiquitination of p27 by SCF^{Skp2} has been achieved in vitro, and appears to not only require interactions between cyclin E and p27, but also with the Cdk-binding subunit Cks1 (Ganoth et al., 2001; Montagnoli et al., 1999).

The SCF complex that targets cyclin E in vivo has resisted discovery until recently. Cyclin E degradation depends in part on phosphorylation at Thr380, as discovered in a yeast-based screen for mutations that stabilize cyclin E (Won and Reed, 1996), and by direct mutation of Cdk sites in cyclin E (Clurman et al., 1996). Because catalytic activity of Cdk2 appears necessary for cyclin E instability, it has been presumed but not proven that Thr380 is a critical autophosphorylation site. Although Skp2 has also been implicated in cyclin E degradation (Nakayama et al., 2000), the mutual antagonism between p27 and cyclin E has made it difficult to disentangle effects on cyclin E from effects on p27. That is, in the presence of excess p27, cyclin E activity is low, and the resultant unphosphorylated cyclin E may be stabilized. Very recently, a new F-box protein called

Fbw7/hCdc4/Ago has been discovered to directly to target phosphorylated forms of cyclin E (Koepp et al., 2001; Moberg et al., 2001; Strohmaier et al., 2001). Interestingly, multiple phosphorylation sites appear to contribute to cyclin E degradation, reminiscent of G1 cyclin degradation in yeast (Strohmaier et al., 2001). Consistent with the observation that cyclin E is elevated in many tumour cell types, Fbw7/hCdc4/Ago is mutated in some cancer cell lines, suggesting that it may play a tumour suppressor function (Moberg et al., 2001; Strohmaier et al., 2001). In addition to its role in the cell cycle, Fbw7/hCdc4/Ago was also discovered as *SEL-10*, a *C. elegans* gene implicated in control of Notch and presenilin signaling (Hubbard et al., 1997).

The Cdc4 Phospho-Degron

Despite success in dissecting the components required for Sic1 ubiquitination, the mechanism whereby Sic1 is recognized by the WD40 repeat domain of Cdc4 has not been thoroughly investigated. Given the precedents for phospho-dependent recognition by SH2 domains and other examples described above, we initially expected that one or more phosphorylation sites on Sic1 and other Cdc4 substrates would be bound by the WD40 repeat domain of Cdc4 in a high affinity interaction with a single phospho-epitope. However, inspection of all known Cdc4 substrates including Sic1 failed to reveal any candidate binding motifs, other than the core Cdk consensus phosphorylation site motif, S/T-P-X-K/R (Figure 6). In a detailed study reported elsewhere (Nash et al., 2001), we have found that the interaction of Cdc4 with multiply phosphorylated substrates differs dramatically from previously described phosphorylation dependent interactions. That is, Sic1 is recognized only upon phosphorylation on many Cdk sites, which cumulatively generate a high affinity interaction (Figure 7). This multi-site phosphorylation dependent interaction imposes a high threshold for Cln-Cdc28 kinase activity in late G1 phase, and even more importantly, appears to confer ultrasensitivity to the Sic1 degradation reaction, thereby allowing precise coordination of DNA replication. The experimental evidence for this model is as follows:

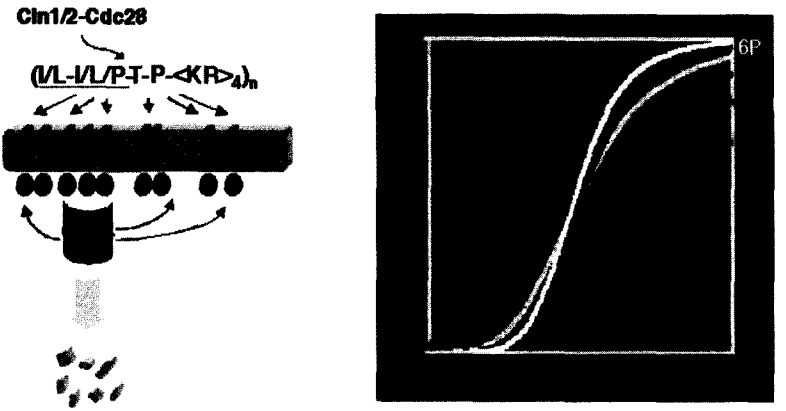

Figure 7. Multi-site phosphorylation confers ultrasensitivity on the Sic1 recognition by SCF^{Cdc4}. Cdc4 efficiently recognizes Sic1 once 6 or more residues are phosphorylated by Cln1/2-Cdc28 kinases. The requirement for phosphorylation on 6 sites causes the degradation pathway to be dependent on the 6th order of kinase concentration, thereby generating a sigmoidal dose-response curve. The increasing degree of cooperativity as a function of number of phosphorylation site required for recognition is illustrated.

1. No single Cdk site in Sic1 is essential for the Cdc4 interaction or degradation in vivo. The rank order of genetic potency for sites in Sic1, as determined by stabilization of Sic1 in vivo is: Thr45 > Ser76 > Thr5 > Thr33 > ≈ Thr2 ≈ Ser69 ≈ Ser80 ≈ Thr173 ≈ Ser191. Re-introduction of 1, 2, 3, 4 or 5 Cdk sites into a version of Sic1 that lacks all nine phosphorylation sites (a mutant called $Sic1^{0P}$), beginning with the genetically most important site (Thr45), fails to restore binding to or ubiquitination of Cdc4 in vitro or degradation in vivo. Strikingly though, restoration of two different combinations of six sites or seven sites restores efficient recognition, ubiquitination and degradation.

2. Phosphopeptides derived from the Thr45 region of Sic1 do not out-compete binding of fully phosphorylated Sic1 to recombinant Cdc4 in vitro. However, a phosphopeptide derived from region surrounding Thr380 of the heterologous substrate mammalian cyclin E ($CycE^{pT380}$) does effectively out-compete phospho-Sic1. The Thr380 site has previously been implicated

in cyclin E degradation in yeast (Clurman et al., 1996; Won and Reed, 1996), a process that turns out to be Cdc4 dependent (Koepp et al., 2001; Nash et al., 2001; Strohmaier et al., 2001). $CycE^{pT380}$-derived phosphopeptides bind with sub-micromolar affinity to Cdc4.

3. By using the $CycE^{pT380}$ phosphopeptide as a biochemical probe, a consensus motif for Cdc4 recognition, termed the Cdc4 Phospho-Degron (CPD), was defined. A high density membrane array of peptides, in which every position of the $CycE^{pT380}$ peptide was varied to each of the 20 naturally occurring amino acids, was assayed for binding to recombinant Cdc4. Surprisingly, the optimal consensus motif I/L-I/L/P-pT-P-<RKY>$_4$ is at odds with the Cdk site consensus S/T-P-X-K/R in that basic residues are disfavoured in the CPD consensus (see Figure 6). A separate derivation of the CPD from a high affinity peptide based on an important phospho-recognition site in Gcn4 (Thr165) yielded a similar consensus sequence, L-P-pT-P-<CR>-<CRKY>$_3$ in which C-terminal basic residues are also disfavoured.

4. Competition binding analysis of a variety of phosphopeptides derived from different Cdc4 substrates revealed a range of affinities. For example, $Sic1^{pT45}$ = 24 μM, $Far1^{pT306}$ = 2.7 μM, $Gcn4^{T165}$ = 0.8 μM. Importantly, multi-site phosphorylation dependent interactions with Cdc4 can be recapitulated with synthetic concatamers of low affinity CPD sites based on either the regions T45 or S76 sites in Sic1.

5. Modeling studies of the Cdc4 WD40 repeat domain revealed a conserved cluster of Arg residues (R467, A 486, R534) predicted to be in spatial proximity on the domain surface. Mutation of these residues, but not other conserved Arg residues, abrogates Sic1 binding in vitro and Cdc4 function in vivo. An analogous cluster of Arg residues is present on Fbw7 (Koepp et al., 2001). As Sic1 itself appears to be devoid of secondary structure, it is unlikely that phosphorylation reveals a masked epitope on Sic1; rather, as for free phosphopeptides, phosphoepitopes on Sic1 probably interact directly with the high affinity site on Cdc4.

6. A single optimal CPD introduced into $Sic1^{0P}$ (called $Sic1^{CPD}$) restores recognition and ubiquitination by SCF^{Cdc4} in vitro and degradation in vivo. However, in individual G1 phase cells, $Sic1^{CPD}$ is eliminated well before cells pass Start, resulting in premature DNA replication, genome instability and lethality in a *cdh1* deletion background.

Multi-Site Phosphorylation and Ultrasensitivity

Taken together these observations suggest that Sic1 is normally recognized only once, although 6 or more Cdk sites are phosphorylated by the Cln1/2-Cdc28 kinases. The incompatibility between the CPD and the Cdk consensus motifs enforces the requirement that multiple low affinity sites have to be phosphorylated before recognition occurs. This requirement probably serves to establish a threshold level of Cln-Cdc28 kinase activity. More importantly, the dependence on multiple phosphorylation events by definition renders Sic1 recognition highly dependent on the concentration of active Cln1/2-Cdc28 kinases (Ferrell, 1996). That is, provided that phosphorylation occurs in a distributive (as opposed to processive) manner, the rate of formation of sextuply phosphorylated Sic1 increases as the 6th order of kinase concentration. Both in vivo and in vitro evidence suggests that Sic1 is indeed phosphorylated in a distributive manner (X. Tang and M.T. unpublished; (Annan et al., 2001)).

The concept of ultrasensitivity was first formulated by Koshland and colleagues to explain the enzymatic properties of metabolic enzymes either in response to phosphorylation or at near saturating substrate levels (Goldbeter and Koshland, 1984; LaPorte and Koshland, 1983; LaPorte et al., 1984). In a classical Michealian response the stimulus-response curve is hyperbolic, such that a 9-fold increase in response requires an 81 fold increase in stimulus. In an ultrasensitive repsonse, the curve is sigmoidal, such that at low stimulus there is little or no response, whereas at the inflection point a small increase in stimulus leads to a very large increase in response. Ultrasensitive reponses can be modeled with the Hill equation, originally derived to describe cooperative effects in enzyme kinetics. Recently, Ferrell has elaborated this concept to entire signaling pathways (Ferrell, 1996; Ferrell, 1999), and provided compelling evidence for a high degree of ultrasensitivity in the complex biological response of *Xenopus* oocyte maturation (Ferrell and Machleder, 1998). Mechanisms that can lead to ultrasensitivity include multi-site phosphorylation, saturation of enzymatic capacity (zero-order ultrasensitivity), saturation of a competing back-reaction, competitive inhibitors, concentration effects upon relocalization and positive feed-back. In extreme cases, such as in oocyte maturation, the response can become virtually switch-like, with Hill

coefficients of 40 or more (Ferrell and Machleder, 1998). The ultrasensitive nature of Sic1 recognition engendered by the need for multi-site phosphorylation serves to sharpen the G1 to S transition by allowing highly coordinated elimination of Sic1, and consequent onset of Clb5/6-Cdc28 kinase activity. Other mechanisms that confer ultrasensitivity may also operate in the Sic1 degradation pathway, including a competing phosphatase back reaction, feed-forward of liberated Clb5/6-Cdc28 activity on Sic1 phosphorylation and competitive inhibition by Cln1/2 autophosphorylation. The net result of these effects is to generate a highly sigmoidal response curve for Sic1 elimination as cells approach Start (see Figure 7).

The Sic1-Cdc4 Interaction: Theoretical Considerations

The observation that Sic1 is recognized only upon phosphorylation of multiple suboptimal CPD sites raises the question as to how this effect is achieved. As modeling and mutagenesis studies suggest the presence of a single high affinity site, one might intuitively suspect that a linear increase in binding sites would give rise to a linear increase in binding affinity. However, in fact a dramatic increase in binding occurs at the transition from 5 to 6 phosphorylation sites (Nash et al., 2001). We therefore derived from first principles the binding characteristics for a linear series of epitopes to a single site. Unexpectedly, this analysis indeed suggests a step-like increase in binding affinity at a threshold number of sites. Below, we present a brief outline of the analysis involved; a detailed model with explicit calculations will be presented elsewhere.

Background: It is a basic consequence of the mathematical form of the familiar Hill equation (see Ferrell, 1996)

$$y = x^n / (C^n + x^n)$$

that a multiplicative amplification of a stimulus will produce a switch-like response. Moreover the higher the value of n, the more switch-like the response becomes (See Figure 8A). This simple fact has been used to hypothesize that an important role of some signalling cascades is to provide this multiplicative amplification, thereby producing a switch. It might be a somewhat less noticed, but equally basic mathematical fact that replacing x^n

with e^x, or for that matter with any similarly shaped, concave function f in the formula f(x)/(f(C) + f(x)) also can produce a sigmoid-shaped curve. (see Figure 8B).

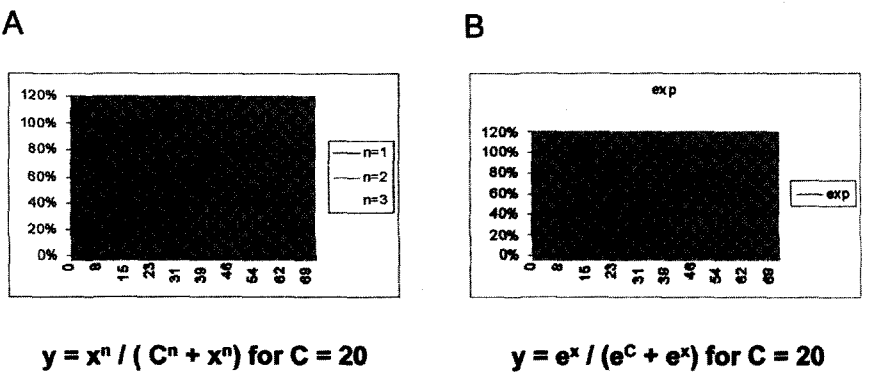

Figure 8. Two routes to ultrasensitivity. A, Cooperativity generates an ultrasensitive dose-response curve. Plots were generated for the Hill equation, $y = x^n / (C^n + x^n)$, for C = 20 for n = 1, 2 and 3. B, An exponential function also generates an ultrasensitive dose-response curve . Plot was generated for the equation $y = e^x / (e^C + e^x)$ for C = 20.

So perhaps it should not come as a surprise that Nature, as often is the case, apparently has found yet another way to exploit this fact. Indeed our analysis based on first principles, suggests that the degree with which Sic1 binds to Cdc4, and which experiments show, as discussed above, have a switch-like sensitivity to the number of phosphorylated binding sites on Sic1, is a function of this type involving an exponential function. The physical mechanism with which this might be accomplished appears not to have been described before.

Discussion

For a single high affinity site one might intuitively expect that a linear increase in binding sites on the substrate would give rise to a linear increase in affinity, since the concentration of sites has increased at a linear rate. To answer this question one needs to understand the effect of several of the sites being situated on the same molecule. In some ways this is analogous to the problem of determining the increase in effective concentration that occurs when two reactants in a reaction are situated on the same larger molecule, and so one might be tempted to try to determine an effective concentration. There is the one major difference, however, that in this case the sites are competitive in that only one can bind to the single high affinity site at a time, yet the net result is a highly cooperative binding. This of course seems like a contradiction. It seems reasonable enough that in a small environment around a Cdc4-Sic1 complex the local, or effective concentration is actually higher than the concentration in the whole solution. This increase in local concentration of binding sites has as a consequence that a Sic1 molecule that has just been dissociated from a Cdc4 has an increased probability of being bound again due to its proximity and multiple binding sites. However even this local concentration increase, and the associated increase in binding probability would only appear to be a linear function of the number of binding sites. But the situation is quite different if one considers the rate of dissociation instead of the rate of association. It is in this rate of dissociation that the non-linearity appears. Since diffusion is a relatively slow process compared to on-rates (Fersht, 1999), there are many opportunities for "re-binding" to occur. Diffusion times are typically measured in micro-seconds for molecular distances while typical waiting times might be measured in nano-seconds at least for two molecules that are in close proximity. Thus the more "re-binding" there is, the less the opportunity for the molecule to completely dissociate itself from its target. This clearly tends to push the value of the equilibrium constant towards binding. Specifically, the probability for a molecule to diffuse away from its partner is related to how much time it has spent in an unbound state; the longer the time-period it has spent without rebinding, the higher the probability that it will be able to escape from the close proximity of the

other molecule. This statement can be made mathematically more precise with an expression of the form

$$\int_0^\infty a(t)f(t)dt$$

where a is the frequency distribution of waiting times and f is the frequency distribution of first passage times. We are suggesting that this effect can be quantified by the fraction of the population of molecules at a given point in time that have spent more than a critical time, unbound but in close proximity to their partner. This fraction of the population is not unexpectedly an exponentially declining function of t, with coefficient of descent equal to the on-rate of the molecule in question. Since the on-rate is easily seen to be a linear function of the number of phosphorylated sites, the proportion of molecules that have waited long enough to have time to diffuse away is an exponential function of this number. Thus a linear increase in the number of binding sites results in a geometric decrease of the time that is available for dissociation through diffusion.

Prospects

We have dissected in detail multi-site phosphorylation-dependent Sic1 recognition by Cdc4 and provided a biological rationale for this mechanism. Our theoretical analysis of the binding reaction should provide the basis for further studies to understand the nature of the binding interaction, in particular how a simple linear increase in binding sites leads to a step-wise increase in binding affinity. This analysis may also illuminate other examples where ultrasensitivity is at play (Ferrell, 1996; Ferrell, 1999). With particular regard to the cell cycle, other instances of multi-site phosphorylation include the regulation of the replication origin firing machinery itself, the interaction of Cdh1 with the core APC particle, and potentially cyclin E and p27 degradation in the mammalian cell cycle (Tyers and Jorgensen, 2000). Given the occurrence of multi-site phosphorylation in many biological contexts (Cohen, 2000), these principles may be widely applicable in situations where all-or none decisions must be made by the cell.

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The 26S Proteasome: A Supramolecular Assembly Designed for Controlled Proteolysis

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Introduction

The 26S proteasome is a huge molecular machine of approximately 2.5 MDa, which degrades protein substrates by an energy-dependent mechanism (for reviews see: Coux *et al.* 1996; Voges *et al.* 1999). It comprises two subcomplexes, the 20S core particle and one or two regulatory complexes, the 19S caps. The 20S complex allows to confine the proteolytic action to a nanocompartment, in which substrates, sequestered from the cellular environment, undergo degradation (Baumeister *et al.* 1997, Lupas *et al.*, 1997). The 19S regulatory complex recruites substrates marked for degradation and prepares them for translocation into the 20S core complex (Lupas *et al.* 1993; Larsen and Finley 1997). The sequence of events encountered by a substrate until it is finally degraded is reflected by a linear arrangement of functional modules within the 45 nm long supramolecular assembly.

Whereas the structure and function of the 20S proteasome have been elucidated in great detail (for review see: Baumeister *et al.* 1998), the 19S regulator is understood only dimly at present. Structural studies are hampered by the low intrinsic stability of this assembly and extensive remodelling, which makes it notoriously difficult to obtain homogeneous preparations. Nevertheless, analyses of yeast, *Drosophila* and human 26S

proteasomes have revealed a common set of 17 to 18 subunits—although a few species-specific differences have been found (Tanaka 1998; Glickman *et al.* 1998b; Ferrell *et al.* 2000; Hölzl *et al.* 2000; Verma *et al.* 2000). These subunits can be assigned to two subcomplexes (Glickman *et al.* 1998a): The 'base' part, which comprises an array of six paralogous AAA-ATPases, and is sufficient to support the degradation of (partially) unfolded proteins and the 'lid' part which provides the link to the ubiquitin system, which, in turn, confers selectivity (Pickart 2000; Wilkinson 2000). Consistent with the lack of an ubiquitin system in archaea and bacteria (Ruepp *et al.* 2000), minimal homo-hexameric AAA ATPase complexes are sufficient for regulating prokaryotic 20S proteasomes (Schmidt *et al.* 1999; Zwickl *et al.* 2000).

In this chapter, we review the current knowledge of the structure, assembly and function of the 20S proteasome and its regulators in prokaryotic and eukaryotic cells.

The 20S Proteasome

20S proteasomes are ubiquitous and essential in eukaryotes (Heinemeyer 2000); ubiquitous but not essential in archaea (Ruepp *et al.* 1998); and rare and non-essential in bacteria (Knipfer and Shrader 1997; De Mot *et al.* 1999). Because of ist relative simplicity the proteasome from the archaeon *Thermoplasma acidophilum* (Dahlmann *et al.* 1989) has played a pivotal role in resolving the structure and enzymatic mechanism of 20S proteasomes (see for example: Hegerl *et al.* 1991; Grziwa *et al.* 1991; Pühler *et al.* 1992; Zwickl *et al.* 1992; Jap *et al.* 1993).

In spite of the differences in subunit complexity, the quaternary structure is highly conserved in 20S proteasomes from all three domains of life. The 28 subunits, 14 of the α-type and 14 of the β-type, are grouped into four seven-membered rings, which collectively form a barrel-shaped complex with a length of 15 nm and a diameter of 11 nm (see Figure). The two adjacent β-subunit rings, enclose the central cavity with a diameter of approximately 5 nm, which harbours the active sites. It is connected via two narrow constrictions with two slightly smaller outer cavities, the 'antechambers', which are formed jointly by one α and one β ring. An axial pore in the α-rings gives access to the antechambers. In most prokaryotic

proteasomes the rings are homomeric and the complex is described by an $\alpha_7\beta_7\beta_7\alpha_7$ stoichiometry. The stoichiometry of eukaryotic 20S proteasomes is $\alpha_{1\text{-}7}\beta_{1\text{-}7}\beta_{1\text{-}7}\alpha_{1\text{-}7}$. Each of the 14 different subunits is present in two copies within one complex and occupies a precisely defined position (Schauer *et al.* 1993).

As anticipated from their sequence similarity, the (non-catalytic) α- and the (catalytic) β-type subunits have the same fold (Löwe *et al.* 1995; Groll *et al.* 1997): a four-layer α+β structure with two antiparallel five-stranded β sheets, flanked on one side by two, on the other side by three α helices. In the β-type subunits, the β-sheet sandwich is closed at one end by four hairpin loops and open at the opposite end to form the active-site cleft; the cleft is oriented towards the inner surface of the central cavity. In the α-type subunits an additional helix formed by an N-terminal extension crosses the top of the β-sheet sandwich and fills this cleft. Initially, the proteasome fold was believed to be unique; however it turned out to be prototypical of a new superfamily of proteins referred to as Ntn (N-terminal nucleophile) hydrolases (Brannigan *et al.* 1995).

Site-directed mutagenesis and the crystal structure analysis of a proteasome-inhibitor complex identified the amino-terminal threonine (Thr1) of *Thermoplasma* β subunits as both, the catalytic nucleophile and the primary proton acceptor (Seemüller *et al.* 1995; Löwe *et al.* 1995).

Proteolytically active β-type subunits are synthesized in an inactive precursor form containing N-terminal extensions of variable lengths, the propeptides, which must be removed posttranslationally to allow the formation of active sites. This process is tied in with the assembly of the 20S proteasome in such a manner, that activation is delayed until assembly is complete and the active sites are sequestered from the cellular environment. Cleavage of the propeptide proceeds autocatalytically, relying on the active-site threonine, and the invariant glycine at position -1 appears to be the prime determinant of the cleavage site (Schmidtke *et al.* 1996; Seemüller *et al.* 1996; Chen and Hochstrasser 1996).

The barrel-shaped architecture of the 20S proteasome allows substrate proteins to be degraded in a processive manner, i.e. without the release of degradation intermediates (Akopian *et al.* 1997). It is noteworthy, that the cleavage sites found when longer peptides or proteins are used as substrates, do not reflect the specificities defined by means of short fluorogenic

peptides (Wenzel *et al.* 1994; Ehring *et al.* 1996). The *Thermoplasma* proteasome for example, when assayed with short fluorogenic peptides displays solely a 'chymotryptic' activity, but chymotryptic cleavage sites are found only rarely in degradation products from polypeptides (Wenzel *et al.* 1994). This suggests that residues beyond P1 (P2, P3, P4, ...) may determine the site of cleavage (for review see: Orlowski and Wilk 2000).

Nevertheless, the peptide products generated by the 20S proteasome fall into a relatively narrow size range of 6-10 amino acid residues. This observation led to the proposal that proteasomes may possess an intrinsic molecular ruler (Wenzel *et al.* 1994). Recent more comprehensive analyses of product lengths, whilst in agreement with an average length of eight residues (±1 residue), showed larger size variations, which are difficult to reconcile with a purely geometry-based ruler which should yield products more focused in length (Kisselev *et al.* 1998; Nussbaum *et al.* 1998; Kisselev *et al.* 1999). Moreover, a reduction in the number of active sites to four or two in mutant yeast proteasomes had little effect on the size of the peptides that were generated (Dick *et al.* 1998). It is therefore unlikely that the distance between active sites is a major determinant of the product size. Studies with synthetic peptides varying in length but displaying the same (repetitive) pattern of cleavage sites indicated that, below a certain threshold in length (<12-14 residues), degradation is decelerated, possibly because the products have a higher probability of exiting the proteolytic nanocompartment (Dolenc *et al.* 1998). Although they might re-enter and be degraded further, this appears to be a slow and inefficient process and therefore products smaller than 12-14 residues accumulate.

Archaeal and Bacterial Activators of the 20S Proteasome

Sequencing of several archaeal genomes revealed the existence of genes with high sequence similarity to the ATPases of the eukaryotic 19S regulator. The deduced 50 kDa proteins have N-terminal coiled-coils, a hallmark of proteasomal AAA ATPases, and C-terminal AAA domains. The *Methanococcus jannaschii* protein was expressed in *E. coli* and purified as a 650 kD complex with nucleotidase activity. When mixed with proteasomes from *Thermoplasma*, degradation of substrate proteins was stimulated up to

25-fold; hence the complex was named PAN, for proteasome activating nucleotidase (Zwickl *et al.* 1999). *Methanococcus* PAN was recently shown to recognize ssrA-tagged GFP and mediate its energy-dependent unfolding and subsequent translocation into the 20S proteasome for degradation (Benaroudj and Goldberg 2000).

Homologs of *Methanococcus* PAN have been found in many but not in all archaeal genomes. No PAN homolog exists for example in *Thermoplasma* (Ruepp *et al.* 2000) and in the more distantly related *Pyrobaculum aerophilum* (S. Fitz-Gibbon, pers. commun.). Therefore, the role of PAN in activating the 20S proteasome must be assumed by different molecules in these organisms, most likely more divergent members of the AAA ATPase family. The complete sequence of the *Thermoplasma* genome revealed three candidate proteins, namely VAT, a two domain AAA ATPase, which is closely related to yeast Cdc48 and human p97 and two one domain AAA ATPases, VAT2 and Lon2 (Ruepp *et al.* 2000). While the latter proteins have not yet been characterized, a chaperone-like activity was demonstrated for *Thermoplasma* VAT (Golbik *et al.* 1999).

A recurring feature of all bacteria possessing genuine 20S proteasomes is the existence of a gene, ARC, encoding a more distant member of the AAA family of ATPases, which is found upstream of the proteasome operons (Nagy *et al.* 1998; De Mot *et al.* 1999). The recombinant ARC ATPase from *Rhodococcus erythropolis* is a complex of two six-membered rings with ATPase activity; like other proteasomal ATPases it has an N-terminal coiled-coil domain (Wolf *et al.* 1998).

The 19S Regulatory Complex

In eukaryotic cells, the 20S proteasome assembles with one or two 19S regulatory complexes (RC) in an ATP-dependent manner to form the 26S proteasome (see Figure 1) (Hough *et al.* 1987; Ganoth *et al.* 1988; Driscoll and Goldberg 1990; Peters *et al.* 1994). The 26S holoenzyme is the most downstream element of the ubiquitin-proteasome pathway.

Slightly differing sets of RC subunits have been reported for different organisms (Udvardy 1993; DeMartino *et al.* 1994; Dubiel *et al.* 1995; Tanaka 1998; Glickman *et al.* 1998b; Hölzl *et al.* 2000) and the abundance

of factors that have been described to associate to the RC in a tissue and devolopment specific manner and with variable affinities, makes it difficult to draw the line between interacting factors, transiently bound subunits or integral components of the RC. However, as the mass of the *Drosophila* RC, approximately 890 kDa, determined by scanning-transmission electron

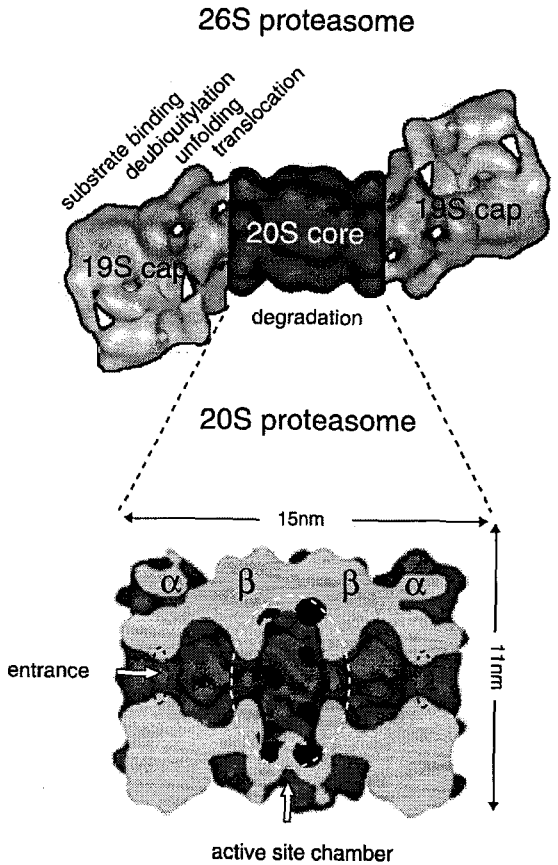

Figure 1. Composite model of the three-dimensional (3-D) structure of the 26S proteasome combining a 3-D reconstitution from electron micrographs of *Drosophila* 26S complexes and the crystal structure of the *Thermoplasma* 20S proteasome (Walz *et al.* 1998). Below, the 20S structure is cut open along the seven-fold axis for display of the three inner compartments; in the central compartment 8 of the 14 active sites are marked in black.

microscopy (STEM) measurements is in good agreement with the summed masses of the 18 individual subunits (932 kDa) identified on 2D gels (Hölzl *et al.* 2000), it seems likely that the catalogue of integral subunits is now complete. The catalogues of the *Drosophila* and yeast 19S subunits are identical, exept for a single subunit (p37A), which seems to be absent from yeast 26S proteasomes.

The 19S regulator of the yeast proteasome can be dissociated into 2 subcomplexes, the 'base' and the 'lid', which are located proximally and distally with respect to the 20S core (Glickman *et al.* 1998a). The complex formed by the base and the 20S proteasome is sufficient for the degradation of non-ubiquitylated protein substrates, but does not mediate degradation of ubiquitylated substrate proteins. From the location of the base in the 26S complex it had been inferred to have a role in substrate unfolding, acting as a 'reverse chaperone', and in controlling the gate in the coaxially apposed α-rings of the 20S particle (Lupas *et al.* 1993). Recently, a chaperone-like acitvity was demonstrated for the base (Braun *et al.* 1999; Strickland *et al.* 2000).

As only the 26S holoenzyme, but not the 20S-base complex degrades ubiquitylated proteins (Glickman *et al.* 1998a; Thrower *et al.* 2000), it appears that recognition, and binding of ubiquitin-tagged substrates is mediated by the eight subunits of the lid subcomplex.

The base is composed of the two largest subunits of the 26S proteasome, S1/Rpn2 and S2/Rpn1, and six paralogous AAA ATPases. The ubiquitin-chain binding subunit S5a/Rpn10 was originally assigned to the base, but there is now consensus that it is located at the base-lid interface. The ATPases perform and regulate some of the basic functions of the 26S proteasome (Lupas *et al.* 1993; Larsen and Finley 1997). First, they are required for the ATP-dependent assembly of 26S proteasomes from the 19S RC and the 20S core (Armon *et al.* 1990; DeMartino *et al.* 1994; Peters *et al.* 1994; Hoffman and Rechsteiner 1996; Verma *et al.* 2000). Second, as attachment of the base complex to the 20S core is sufficient to activate peptidase activity the ATPases are believed to be involved in the gating of the α-ring channel and thus in controlling access to the proteolytic core. Third, as the base complex and its evolutionary ancestor PAN have been shown to have chaperone-like activity *in vitro*, the ATP-dependent unfolding of substrates can be attributed to the ATPases, acting in a 'reverse

chaperone' mode (Lupas *et al.* 1993; Zwickl and Baumeister 1999). Finally, it is possible that unfolding and translocation are mechanically coupled (a 'pushing' mechanism), and therefore substrate translocation is dependent on the ATPase activity.

The two largest subunits of the base complex, S1/Rpn2 and S2/Rpn1 have significant sequence similarities (~20% identity) and are likely to have a common ancestor (Tsurumi *et al.* 1996). Both contain leucine-rich-like repeats (LRR) at their C termini, similar to repeats found in the BimE subunit of the anaphase-promoting complex (APC) or cyclosome (Lupas *et al.* 1997), as well as motifs rich in alternating lysine (K) and glutamate (E) residues (KEKE) (Realini *et al.* 1994); both motifs are supposed to be involved in protein-protein interactions.

As the deletion of S5a/Rpn10 facilitates dissociation of the RC into base and lid, it is assumed to be located at the interface; this is consistent with observations that it interacts with multiple subunits in the base and in the lid (Ferrell *et al.* 2000). Interestingly, S5a/Rpn10 is the only subunit of the 19S complex that is also present in significant amounts in free monomeric form (Haracska and Udvardy 1995; van Nocker *et al.* 1996).

The lid has been isolated as a discrete complex from yeast and from human erythrocytes (Glickman *et al.* 1998a; Henke *et al.* 1999); it comprises eight subunits, S3/Rpn3, Rpn5, S9/Rpn6, S10a/Rpn7, S11/Rpn9, S12/Rpn8, S13/Rpn11, and S14/Rpn12. Intriguingly, each of the individual subunits has a counterpart with extended sequence similarities in the COP9 signalosome, a multiprotein complex involved in signal transduction (Seeger *et al.* 1998).

In addition to the RC subunits detected in all eukaryotic organisms investigated so far, a few subunits seem to be species-specific. A member of the ubiquitin carboxy-terminal hydrolases (UCH) (Wilkinson 2000), has been found in *Drosophila* (p37A) (Hölzl *et al.* 2000), *S. pombe* (Uch2) (Li *et al.* 2000), and human (UCH37) (Li *et al.* 2001), but not in *S. cerevisiae* 26S proteasomes. The *Drosophila* p37A subunit was mapped to the base-lid interface in close vicinity of the putative location of S5a/Rpn10 (Hölzl *et al.* 2000).

Structural Features of the 26S Proteasome

On electron micrographs, 26S proteasomes appear as elongated, dumbbell-shaped particles. Samples from a variety of different organisms all show a mixture of 20S core particles 'capped' with either one or two RC(s) (Peters *et al.* 1993; Yoshimura *et al.* 1993; Fujinami *et al.* 1994). Averages obtained from negatively stained preparations reveal a characteristic 'dragon head' motif (Rechsteiner 1998) with the RCs facing in opposite directions in the double-capped particles. Thus they appear to reflect the underlying C2 symmetry of the core particle; a more rigorous analysis of inter-image variations has shown, however, that there is a small but significant deviation from exact C2 symmetry. Moreover, the 19S caps appear not to be in a fixed position with respect to the 20S core particles, but to undergo a peculiar up and down ('wagging') movement (Walz *et al.* 1998); hitherto the functional relevance of this movement is not clear. Furthermore, it cannot be ruled out that the observed wagging describes the real movements inadequately; adsorption of the 26S complex to the carbon film prior to negative staining may severely restrain movements of the 20S and 19S subcomplexes with respect to each other. In fact, a large variety of states is observed with unsupported 26S complexes embedded in vitreous ice. Amongst them are double-capped complexes which appear nearly mirror-symmetric, which is indicative of a rotary movement between the pseudo-sevenfold α-ring of the 20S particle and the pseudo-sixfold ATPase ring in the base part of the 19S RC (Kapelari et al., in preparation).

Such a symmetry mismatch has analogies in other rotating molecular machines. For the bacterial ClpAP system it has been shown that small rotational increments of 8.6 degrees are sufficient to bring six- and seven-fold rings into (pseudo-)equivalent positions (Beuron *et al.* 1998). Whether or not a rotary movement has a role in the unfolding and translocation of target proteins is currently a matter of speculation.

Conclusions

Structure, assembly and enzymatic mechanism of the 20S complex have been elucidated, but the functional organization of the 26S proteasome is

understood only dimly at present. The constituent subunits of the 19S complex have been identified, however, specific functions have only been assigned to a few. The molecular details of the distinct steps of substrate recognition, unfolding and translocation *en route* to the 20S core complex where degradation takes place remained elusive so far.

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Mechanisms and Regulation of Ubiquitin-Mediated, Limited Processing of the NF-κBα Precursor Protein p105

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Summary

In most cases, target substrates of the ubiquitin proteolytic system are completely degraded. In several exceptions, such as the first step in the activation of the transcriptional regulator NF-κB, the substrate-the precursor protein p105—is processed in a limited manner to yield p50 (residues 1-435), an active subunit of the heterodimeric factor. p50 is derived from the N-terminal domain of p105, whereas the C-terminal, ankyrin repeat-containing domain, is degraded. The mechanisms involved in this unique process have remained largely obscure. A Gly-rich region (GRR) in the C-terminal domain of p50 acts as a "stop" signal and interferes with processing of the ubiquitinated precursor by the 26S proteasome. In addition, p105 contains two distinct targeting motifs that act under different physiological conditions. In the resting cell, Lys residues 441 and 442 serve as ubiquitination targets, whereas the neighboring downstream acidic sequence (residues 446-454), can function as a ligase recognition motif. Following IκB kinase (IKK)-mediated phosphorylation of Ser residues in the C-terminal domain of p105 (amino acids 918-934), the $SCF^{\beta-TrCP}$ ubiquitin

ligase is recruited, and ubiquitination of yet unknown Lys residues leads to accelerated processing. Ubiquitin conjugation and subsequent processing of a series of precursors of p105 that lack the C-terminal signaling domain, but still contain the ankyrin repeat domain, is progressively inhibited with increasing number of repeats. Inhibition is due to docking of active NF-κB subunits to the repeats. Inhibition is alleviated by phosphorylation and ubiquitination of the C-terminal signaling domain that result in degradation of the ankyrin repeat domain and release of the anchored subunits. A model is proposed that may explain the requirement for two sites for p105 processing. Under basal conditions, p50 is generated cotranslationally or via slow processing of mature p105 that involves the mid-molecule site. Following signaling, the C-terminal site is involved in rapid processing/degradation of the mature molecule with release of a large amount of stored, transcriptionally active subunits.

Introduction

The NF-κBα dimeric transcription factors play key roles in basic processes such as regulation of the immune and inflammatory responses, development and differentiation, malignant transformation and apoptosis (1). Certain active subunits of NF-κBα are generated from inactive precursor molecules via limited, ubiquitin- and proteasome-mediated processing. One established case is that of p50 that is generated from the p105 precursor (2,3). p50 is derived from the N-terminal domain of the molecule, while the C-terminal, ankyrin repeat-containing domain (IκBγ), is degraded (4). The processed subunits typically heterodimerize with members of the rel family of transcription factors such as p65 (RelA) to generate the active heterodimeric p50–p65 transcription factor. Binding of a third protein, a member of the IκB family of inhibitors, sequesters the transcription complex in the cytosol. Following cellular stimulation, activated IKKs phosphorylate IκB on specific Ser residues (residues 32 and 36 in IκBα). Phosphorylation leads to recruitment of the $SCF^{\beta\text{-}TrCP}$ ubiquitin ligase complex, polyubiquitination and subsequent degradation of the inhibitor by the 26S proteasome. Following degradation of IκB, the heterodimer is translocated into the nucleus where it initiates specific transcription (reviewed recently in Ref. 5).

The ubiquitin pathway is involved in regulation of broad array of basic cellular processes, such as cell cycle progression and division, differentiation and development, and the immune and inflammatory responses. Involvement of the system in these processes is mediated via specific destruction of short-lived regulatory proteins such as cyclins, transcriptional activators, and cell surface receptors, and generation of antigenic peptides presented on class I MHC molecules. Degradation of a protein by the system involves two successive steps: (a) formation of a polyubiquitin chain that is covalently anchored to the target substrate, and (b) degradation of the tagged protein by the 26S proteasome. Conjugation of ubiquitin involves three steps: (a) activation of ubiquitin by the ubiquitin-activating enzyme, E1, (b) transfer of the activated moiety to a member of the ubiquitin-carrier protein—E2—family of enzymes (also known as ubiquitin-conjugating enzymes, UBCs), and (c) further transfer of the activated ubiquitin moiety from E2 to the target substrate that is specifically bound to a member of the ubiquitin-protein ligase family of proteins, E3. Subsequent processive transfer of additional activated ubiquitin molecules and their conjugation to previously attached moieties generates a polyubiquitin chain that serves as a degradation signal for the proteasome. The initial binding of the substrate to E3 plays an essential role in specific substrate targeting. Several classes of E3s have been described, among them are the SCF complexes that recognize phosphorylated substrates involved, for example, in cell cycle regulation and the immune and inflammatory response. These tetrameric complexes are composed of Skp1, Cullin1 and Rbx1/Roc1 that are common to all SCFs, and a variable F-box protein that serves as the substrate-recognizing subunit, E3 (for recent general reviews on the ubiquitin system and the proteasome, see for example Refs. 6 and 7, respectively; for recent reviews on E3s and SCF complexes, see for example Refs. 8 and 9, respectively). The F-box protein involved in recognition of phosphorylated IκBα is human β-TrCP. Recognition of the inhibitor is mediated via the sequence [DS(P)GLDS(P)]. A similar motif is probably involved also in the recognition of human β-Catenin and HIV-Vpu (see below).

The mechanisms involved in limited processing of the p105 precursor protein have been partially elucidated. Lin and Ghosh (10) have demonstrated that a GRR in the middle of the molecule (residues 376-404)

is required for processing. Fan and Maniatis (4) have shown that a truncated form of p105, p60, can be processed to p50. Lin and colleagues (11) have shown that p105 can be processed cotranslationally, and synthesis of the complete molecule is not required for generation of p50. Several single residues that reside upstream to the GRR and are involved in proper folding p50, are also essential for processing, most probably via maintaining a tight folded structure that cannot enter via the narrow proteasomal orifice, a process that requires, most probably, unfolding (12). Taken together, these studies suggested that all the motifs that are required for basal processing that occurs most probably immediately distal to residue 435, are contained within the first ~550 amino residues. Other studies have suggested a role for phosphorylation of the C-terminal domain of p105 in regulated, signal-induced processing/degradation of the molecule (see for example Refs. 13,14). Heissmeyer and colleagues have shown that IKK-mediated phosphorylation of Ser residues localized to a region that spans amino acid residues 922-933 leads to rapid degradation of p105 (15). Thus, it appears that processing of p105 can occur under both basal and activated conditions, and that each process is probably mediated via distinct structural motifs and catalyzed by different E2 and E3 enzymes. The enzymes and their mode of action had remained obscure until recently.

Results

The Gly-Rich Region (GRR) is a Processing "Stop" Signal

It has been reported that the GRR that spans amino acid residues 476-304 in human p105 , is an important structural motif required for the generation of the p50 subunit of NF-κB (10). However, the mechanisms involved in this unique reaction have remained enigmatic. Since degradation of a protein via the ubiquitin pathway involves two steps, conjugation of ubiquitin and proteasomal degradation of the tagged substrate, it was important first to identify the step affected by the GRR.

We have shown that conjugation of p105 is not affected by GRR either *in vitro* or *in vivo*, and it appears to interfere with the function of the 26S proteasome (16). This interference predicts two related assumptions: (a) that

processing involves recognition motif(s) that reside in C-terminal half of p105 that is degraded, and (b) that the GRR protects/stabilizes the newly formed p50 subunit. To test the second notion, we generated two p50 derivatives, one that contains the GRR and one that lacks this domain. Both proteins share the C-terminal domain of native p50 (residues 405-435). The GRR-containing protein is significantly more stable then its mutated counterpart both *in vitro* and *in vivo*. Thus, it appears that the GRR is involved in the generation of p50 by serving as processing "stop" signal and as a stabilizing element of the cleaved fragment.

To analyze structure/function relationship of different elements within the GRR, we studied the function of different deletion and point mutants. The human p105 GRR contains 19 Gly residues (out of 29 residues in total; 376-GGGSGAGAGGGGMFGSGGGGGGTGSTGPG-404), two of which are interspaced by Ala (380-GAGAG-384). Deletion analysis revealed that only 6 (382-GAGGGGMFGS-391) Gly residues are sufficient to promote, at least partially, generation of p50. Importantly, the Ala residue is essential (16).

Since a small GA-containing sequence appears to function as a processing signal, it was also important to examine its "universality" as a transferable processing signal. Its transfer to bona fide substrates of the ubiquitin system, including MyoD, p53, or ODC (that is targeted by the proteasome without prior ubiquitination), and the *D. melanogaster* Dorsal (which is closely related to p105), did not render these proteins susceptible to processing. Even its transfer within p105 (to position 604-613) did not yield a processing product (16). Taken together, these findings suggested that in addition to the GRR, processing requires also an additional motif(s) that resides in a defined distance, up- or downstream to the GRR.

A Region Downstream to the GRR is Required for Processing

In our attempt to identify this putative additional motif(s), we noted a sequence that resides downstream to the GRR and that is highly homologous to the ubiquitination and E3-binding domain of IκBα (Table 1). To test the possible role of this motif in p105 processing, we generated two p105 mutants: (i) K441,442R in which we substituted with Arg the two Lys residues that are homologous to IκBα Lys 21 and 22, and (ii) p105δ446-454

that lacks the acidic domain and Ser 450, and which is similar to the IκBα signaling motif. Our data show that the two Lys residues are important for ubiquitination of p105, while residues 446-454 are independently important for ubiquitination and processing, possibly via binding of the E3 (16). However, unlike Ser32 of IκBα, processing of p105-S450A is indistinguishable from that of the WT protein, suggesting that phosphorylation of this residue is not necessary for processing (16).

To examine the biological consequences of the alterations in the different processing signals, we transfected COS-7 cells with the WT p105, p105-δGRR and p105-K441,442R, and monitored binding of labeled κB probe in electromobility shift assay (EMSA) as a measure for the formation of biologically active p50. While WT p105 generates active NF-κB that specifically binds the labeled probe, p105-δGRR does not generate any binding activity, and the activity generated by p105-K441,442R is markedly reduced (16).

Table 1. Comparison of the ubiquitination/recognition domains of human p105 (residues 441-454) and IκBα (residues 20-39)

Human p105:	440- SKK***D***P***E***GC***D***K**S*****DD*** -	454
	||+ +	
Human IκBα:	20- LKK***E***RLL***DD***RH***D*****S**GL***D*****S**MK***D***-	39

The pair of lysine residues that serve as ubiquitination sites in both molecules are marked by vertical lines. Two overlapping acidic residues are marked by +. Ser residues are larger and bolded, whereas all acidic residues are bolded and italicized (6 in IκBα and 5 in p105).

IKK-Mediated Signal-Induced Phosphorylation of the C-Terminal Domain of p105 Recruits the $SCF^{\beta\text{-}TrCP}$ E3 Complex Which Results in Accelerated Processing/Degradation of p105

Since deletion of residues 441-454 did not abolish processing completely (16) and since signal-induced phosphorylation that could not possibly involve residues 446-454 (see above) was reported to regulate p105 processing (13,14; see above), we predicted that an additional, signal-

regulated motif may be involved in recognition of p105 by the ubiquitin system. Since the C-terminal domain contains an IKK phosphorylation site that is involved in regulated processing/degradation of p105 (15), we decided to dissect the mechanism(s) that underlie its involvement in the process. Quantitative analysis revealed that deletion of residues 446-454 or of residues 918-934 that comprise the IKK phosphorylation site, reduces processing by ~80%. Processing was abolished almost completely in a p105 molecule that lacks both domains (17).

Since IKKs can modify the C-terminal domain of p105 (15), it was important to test whether the modification affects processing. Expression of constitutively active IKKβ stimulates significantly processing of p105 (17). Quantitative analysis revealed that almost all the precursor protein disappeared. While most of it was processed to p50, a significant part was completely degraded. The kinase stimulated processing of p105-WT and p105-δ446-454 to the same extent. In contrast, it did not have any effect on processing of p105-δ918-936 (17). Thus, it appears that IKKβ stimulates p105 processing via its activity on the C-terminal domain of the molecule.

To demonstrate that IKKβ phosphorylates the C-terminal domain of p105, we reconstituted the phosphorylation system *in vitro*. In the presence of ATP and IKK, only ^{35}S-methionine-labeled WT but not C-terminal-deleted p105 was converted into a slower migrating form, which could be re-converted into the faster migrating form following addition of alkaline phosphatase. Similar results were obtained using [γ-^{32}P]ATP where a strongly phosphorylated form was obtained only when the WT protein was present in the reaction mixture. These *in vitro* experiments show that IKKβ modifies p105 on Ser and/or Thr residues in the region that spans moieties 918-934 (17).

At this stage, it was important to identify the E3 involved in recognition of the phosphorylated C-terminal region of p105. Obvious candidates were members of the SCF family of ligases such as $SCF^{\beta\text{-}TrCP}$ and SCF^{Skp2} that recognize phosphorylated substrates (reviewed recently in Ref. 9). Transfection of cells with the dominant negative E3 δF-box β-TrCP1, inhibited significantly IKKβ -dependent processing of p105-WT. In contrast, processing of p105-δ918-934 was not affected. Similarly, transfection with δF-box Skp2 had no effect (17). Based on these findings, we concluded that TrCP may serve as the E3 that recognizes the

phosphorylated C-terminal domain of p105, and is involved in phosphorylation-mediated processing of the molecule. To further explore the role of β-TrCP in p105 recognition, we utilized the phospho-peptide that spans the phosphorylation domain of IκBα. This peptide binds specifically to TrCP and inhibit its activity towards IκBs α and β (18). The peptide inhibited significantly conjugation and processing of both WT and δ446-454 p105s in a cell free reconstituted system, but had no effect on the conjugation of δ918-934-p105. The experiments in which we used the dominant negative δF-box β-TrCP and the peptide provided only indirect evidence for the involvement of the enzyme in p105 processing. A more direct proof for the involvement of β-TrCP in ubiquitination of p105 came from co-immunoprecipitation experiments, where we demonstrated physical association between TrCP1 and p105 which is increased several-fold following TNFα stimulation of cells. Only p105-WT that was phosphorylated by IKK, could be precipitated by TrCP. p105-WT that was incubated in the absence of the kinase or p105-δ918-934 that was incubated in the presence of the kinase failed to associate with TrCP. Last, it was important to demonstrate directly that β-TrCP can conjugate ubiquitin to C-terminally phosphorylated p105 and promote its processing. Tetrameric (Skp1/Cullin1/β-TrCP/Roc1) $SCF^{\beta\text{-}TrCP}$ complex conjugated *in vitro* phosphorylated p105-WT. A substrate that was not phosphorylated prior to the incubation with the ligase, was not conjugated (17). All these experiments firmly established the role of β-TrCP in recognition of the phosphorylated C-terminal domain of p105, a finding that was corroborated later in an independent study (19). To dissect the structure of the C-terminal motif of p105 targeted by TrCP, we monitored initially conjugation of WT, δ446-454, and δ918-934 p105s in a reconstituted cell free system. The WT and δ446-454 proteins were efficiently conjugated by the SCF complex. In contrast, p105-δ918-934 did not generate high molecular mass adducts. To specifically identify the residues that play a role in recognition of phosphorylated p105, we substituted Ser residues 921, 923, and 932 with Ala. These replacements abolished the ability of TrCP to catalyze conjugation, strongly suggesting that modification of one or several of these residues is essential for targeting by the kinase and for the subsequent recruitment of the ligase complex. Later findings by Heissmeyer and colleagues demonstrated that the recognition motif—922-DSVCDS-927—is

similar to the motif that is targeted by TrCP in IκBα (31-DSGLDS-36), β-catenin (DSGIHS) and HIV-Vpu (DSGNES)(19), and has the general structure of DSGΘXS.

While recognition of the phosphorylated C-terminal domain is mediated by $SCF^{\beta\text{-}TrCP}$, it was not clear whether this E3 is also involved in recognition of the upstream acidic domain. Since the SCF complex is involved in targeting phosphorylated substrates, its involvement in targeting the upstream acidic domain is highly unlikely. In cells, dominant negative δF-box β-TrCP has only a minor effect on processing of p105-δ918-934. Also, processing of the C-terminally deleted mutant is not affected by the inhibitory phosphopeptide. Furthermore, WT and δ446-454 p105s are equally conjugated by the SCF complex *in vitro*. To test a possible role for an additional, yet unidentified, E3 in targeting the acidic domain, WT and the C-terminal deleted p105s were incubated in the presence of crude HeLa extract. Unlike the purified SCF complex that could not conjugate p105-δ918-934, both proteins were efficiently conjugated in the crude extract, suggesting that cells contain an additional, acidic domain-recognizing E3. While indirect, these finding strongly suggests that p105 is conjugated by at least two ligases, recognizing two distinct motifs. It is not known whether Lys 441 and 442 are shared by the two E3s as ubiquitin anchors. More important, it is not clear why processing requires two independent motifs, and whether the two motifs function under different physiological conditions.

Regulation of Signal-Induced Processing of NF-κB P105 and the Requirement for Two Distinct Targeting Sites: Processing of p105 is Inhibited by Docking of p50 Active Subunits to the Ankyrin Repeat Domain, and Inhibition is Alleviated by Signaling via the C-Terminal Phosphorylation/Ubiquitin-Ligase Binding Domain

As has been clearly demonstrated, p105 has two distinct ubiquitin system targeting motifs, an acidic domain that contains also the Lys residues essential for ubiquitin anchoring, and a C-terminal phosphorylation/E3-binding domain. An important, yet unresolved, question involves the biological rationale behind the evolution of these two sites. To resolve this

problem, one has to unravel the physiological conditions under which the two sites are distinctly utilized.

We noted that under basal, non-stimulated conditions, the efficiency of processing of p105 in both cell free system and intact cells is not efficient. Typically, 10-20% of the precursor molecules are processed (16,17,20). It has been shown that active NF-κB subunits dock to the C-terminal domain of p105 and probably inhibit its processing (21). Therefore, we wanted to test the hypothesis that the efficiency of processing is inversely correlated with the number of ankyrin repeats to which the active subunits dock, and that inhibition of processing is mediated via blocking of recognition of the constitutive site by the ubiquitin system. Further, we wanted to test whether alleviation of inhibition and supply of transcriptionally active subunits requires signal-induced targeting of p105 via the C-terminal domain of the molecule. To test this hypothesis, we constructed a series of p105 deletion mutants that contain an increasing number of ankyrin repeats, yet lacking the C-terminal domain. *In vitro* processing of the different C-terminally deleted p105 precursors was progressively inhibited with increasing number of ankyrin repeats. In contrast, p105-WT that contains seven ankyrin repeats, but also an intact C-terminal targeting domain, was processed as efficiently as p105 that does not contain any ankyrin repeat (22). Similarly, conjugation of ubiquitin to the different truncated species of p105 was also progressively inhibited with increasing number of ankyrin repeats. Again, the efficiency of conjugation of the WT protein that contains the C-terminal signaling domain was similar to that of a p105 protein that does not contain any ankyrin repeat (22).

We surmised that the progressive inhibition in processing and conjugation that has been observed with increasing number of ankyrin repeats, is due to the presence of free p50 and other active NF-κB subunits in the HeLa cell extract in which the reactions were carried out. To corroborate this assumption directly, we overexpressed in cells a mutant species of p50 that is slightly larger then the native molecule (so it migrates electrophoretically slower then the native processed product and can be distinguished from it), yet it cannot be further processed as it does not contain the downstream elements required for processing to occur (16 and see above). Overexpressed p50 selectively inhibits processing of WT-p105, but not of an ankyrin repeat-free p105 (22). The finding that processing of

p105 that lacks ankyrin repeat is not affected by the overexpressed p50 rules out the possibility that the expressed p50 interferes with processing by sequestering certain required components, enzymatic elements of the ubiquitin-conjugation machinery, for example. Similar to the *in vivo* experiment, addition of exogenous, bacterially expressed p50 to a cell free proteolytic system resulted in inhibition of processing of p105 precursor molecules that contain more then four ankyrin repeats. Not surprisingly, processing of an ankyrin repeat-free precursor is not inhibited by increasing concentration of exogenous p50. The conclusion that four ankyrin repeats are required in order to confer inhibition is based on the observation that while processing of a p105 protein that contains three ankyrin repeats is not inhibited by exogenous p50, that of p105 that contains four or more is strongly inhibited (22). To further dissect the mechanism(s) that underlie inhibition of processing, we examined the effect of exogenously added p50 on the efficiency of conjugation of the different ankyrin repeat-containing precursors. In contrast to the results of the processing experiment, conjugation of all the different species of p105 is not affected by exogenous p50, suggesting that the anchored subunits interfere, similar to the interference of the GRR, only with the downstream proteasomal activity.

We have shown that IKK-mediated phosphorylation of specific Ser residues within the C-terminal domain of p105 leads to recruitment of the $SCF^{\beta\text{-TrCP}}$ ubiquitin ligase with subsequent polyubiquitination and rapid processing/degradation of the precursor molecule (17). Heissmeyrer and colleagues (15) have shown that IKK-mediated degradation of p105 probably releases docked p50 that interacts, in its free form, with Bcl-3 to generate the trimeric p50/p50/Bcl-3 active transcription factor. We hypothesized that the mechanism that underlies alleviation of inhibition of processing and liberation of the docked inhibitory subunits, involves C-terminal, IKK- and β-TrCP-mediated targeting of p105. To test this hypotheses directly, we transfected cells with p105-WT in the absence or presence of p50 along with active or inactive IKKβ. As observed previously, processing of p105 is completely inhibited by the co-expressed p50. Importantly, the inhibition is partially alleviated by expression of a constitutively active IKKβ, but not by expression of a catalytically inactive enzyme. As expected, the kinase had no effect on inhibition of processing of the C-terminally deleted p105 (22). Last, it was important to examine the

role of β-TrCP in the kinase-mediated alleviating effect. We used δF-box β-TrCP, a dominant negative species of the ligase (see above). The IKK-mediated alleviation was abolished by the concomitant expression of δF-box β–TrCP.

Discussion

We have shown that p105 is targeted for processing/degradation by two distinct ubiquitin system recognition motifs. The first motif, which resides downstream and adjacent to the GRR, contains two Lys residues that serve as ubiquitin anchors and a downstream acidic sequence that can serve as an E3-binding site. This motif is most probably involved in basal/constitutive processing/degradation that occurs in resting cells under non-stimulated conditions, and provides the cell with the low amount of p50 required for its activity under these conditions. Processing of p105 under these conditions may occur cotranslationally, and this site may be involved this unique process. The second—C-terminal—recognition motif undergoes signal-induced IKK-mediated phosphorylation with subsequent recruitment of the $SCF^{\beta\text{-}TrCP}$ ubiquitin ligase, polyubiquitination and accelerated processing/degradation of p105. We have shown that the two motifs are targeted via two different E3s, and most probably, via distinct E2s as well (unpublished).

Several proteins have been described that are targeted following recognition of two distinct motifs and conjugation enzymes. Among them are p53 that is targeted by Mdm2 following DNA damage (23) and by the E6/E6-AP ligase complex in high risk, human papillomavirus-transformed cells (24). The yeast mating type transcriptional regulator MATα2 is targeted by two motifs, Deg1 and Deg2, and two E2 enzymes, Ubc6 and Ubc7 (25), however the identity of the E3s and physiological significance of the two signals have remained obscure. Similarly, the model protein lysozyme is targeted by E2-14kDa/E3α following recognition of the N-terminal amino acid residue (N-end rule pathway) , but also by members of the UbcH5 family and a yet to be identified E3 that recognize a mid-molecule, downstream signal (26). Here too, the role of the two sites have not been elucidated. In the case of p105, the physiological relevance of the

involvement of two sites in processing/degradation of the precursor molecule, has also remained obscure.

To resolve the problem of the relevance of the two processing-directing sites in p105, we have shown that processing is regulated by endogenous free NF-κB subunits that bind to the ankyrin repeat, C-terminal domain of the molecule (designated also IκBγ) and inhibit processing of the precursor. Inhibition is alleviated by IKK phosphorylation that leads to $SCF^{\beta\text{-TrCP}}$ recruitment, rapid polyubiquitination, and efficient degradation of C-terminal domain with release of the docked subunits and an additional p50 subunit that originates from the processed precursor.

Based on these results, we propose a model attempting to explain the requirement for two different distinct recognition signals for processing/degradation of p105 under physiological conditions. According to the model, a nascent p105 polypeptide chain can be initially processed cotranslationally. Processing may require the basal/constitutive recognition motif. The p50 that was generated under these conditions is docked to an emerging ankyrin repeat domain in a p105 molecule that has not been processed yet. Docking of several additional/other free NF-κB subunits hinders recognition of these p105 molecules by the proteasome and may halt further cotranslational processing. The completely synthesized p105 along with the docked subunits serve as an inactive storage for these subunits. Following cell stimulation, the C-terminal domain is phosphorylated. This modification leads to recruitment of the $SCF^{\beta\text{-TrCP}}$ ubiquitin ligase which results in polyubiquitination and subsequent processing/degradation of p105 with release of the docked molecules and an additional p50 subunit generated from the precursor. These subunits serve as a source for an active transcriptional regulator in stimulated cells. Several experimental lines of evidence lend support this model, including progressive inhibition of processing with increasing number of ankyrin repeats, and alleviation of inhibition that requires an intact C-terminal domain, IKK and β-TrCP E3. However, several other important problems remain unsolved. The mechanism(s) that underlies the selection of p105 molecules that undergo cotranslational processing from those that are synthesized to completion is not known. It is possible that cotranslational processing is the default mechanism that provides the resting cell with the small amount of p50 required for its basal needs. This occurs following stress/stimulation when

all the endogenous p50 has been depleted. Once a small amount of p50 is synthesized, it binds to the emerging nascent p105 chain and inhibits further generation of p50 which will occur now only following stimulation and targeting of the C-terminal domain. Such an internal, *cis* inhibitory, mechanism could have also played a role in the evolution of the second, signal-induced C-terminal phosphorylation domain.

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Regulation of Receptor Tyrosine Kinases by Ubiquitination

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Abstract

Growth factors and their transmembrane receptor tyrosine kinases play essential roles in survival, proliferation and migration of both normal and tumor cells. An example is provided by the ErbB/HER family of receptors and the multiple neuregulin and EGF-like ligands. The major route leading to negative regulation of signaling downstream of these receptors involves endocytic removal of ligand-receptor complexes from the cell surface. Ubiquitination of receptor tyrosine kinases such as ErbBs plays a central role in their down-regulation, therefore research into the mechanism and role of receptor ubiquitination is tightly coupled to that of receptor endocytosis. Apparently several distinct endocytic itineraries exist: A constitutive pathway acts to maintain a steady state of signaling-competent receptors. In contrast, ligand stimulation and kinase activation triggers a rapid course of receptor endocytosis, resulting in signal termination. This pathway favors ErbB-1 over the other ErbB-family members. A third pathway involving chaperones and co-chaperones displays preference for ErbB-2, the most oncogenic member of the ErbB/HER family. Ubiquitination at or close to the plasma membrane may be a common feature of all three pathways. However, the exact endocytic route and the final degradative destination, namely the lysosome or the proteasome, appear to significantly differ. Additionally, these pathways are

distinguishable in terms of the sorting mechanisms, the effect of kinase activity, and the involvement of specific ubiquitin ligases. Molecular mechanisms underlying degradation of growth factor receptors by both the ligand-induced pathway and the by chaperone-mediated route are discussed and open questions are identified. Detailed understanding of the complex machineries regulating endocytic inactivation of growth factor receptors holds promise for cancer therapy: antibody-induced endocytosis of ErbB-1 and ErbB-2 effectively blocks some types of human cancer, and low molecular weight drugs that target ErbB receptors to destruction through the stress-related pathway are currently being tested in patients.

Introduction

Ubiquitination of some membrane proteins, including receptor tyrosine kinases, serves to regulate the level of the protein presented to the extracellular milieu at the plasma membrane. Thus, control of the level of plasma membrane proteins is a method of regulating the 'awareness' of a cell to its surrounding, and of the capability of a cell to transduce extracellular stimuli to intracellular action. In this manner, a prominent role is addressed to ligand-dependent ubiquitination and down-regulation of receptors in modulation and termination of growth factor signaling [for a recent review see (Wiley and Burke 2001)]. In addition to the ligand-induced mechanism of receptor down regulation, the use of chaperone inhibitors has revealed a distinct cellular mechanism that regulates the level of surface receptors [reviewed in (Neckers et al. 1999b)]. This system appears to act in stabilizing certain cellular proteins, including some receptor tyrosine kinases (RTKs). Misfolded proteins are recognized by the monitoring system, which directs them for refolding. Alternatively, severely unfolded proteins are destined for ubiquitination and degradation. In addition, perturbation of the monitoring system, by use of chaperone inhibitors, or upon stress, also results in ubiquitination and degradation of its client proteins.

Receptor tyrosine kinases are involved in a myriad of signaling pathways, and active mutants promote oncogenesis in animals and in human [reviewed in (Blume-Jensen and Hunter 2001)]. All RTKs are characterized by an extracellular ligand-binding domain, a single transmembrane domain,

and an intracellular domain, which is sub-divided into a juxtamembrane domain, a tyrosine kinase domain, and a carboxy-terminal tail. On the tail reside multiple tyrosine residues, which undergo phosphorylation upon receptor activation, and act as inducible docking sites for downstream effector molecules. RTKs are activated upon ligand binding, which results in receptor dimerization, followed by activation of the kinase domain. The activated kinase phosphorylates the carboxy-terminal tyrosine-based docking sites, followed by phosphorylation of receptor substrates. A major model system for RTKs has been the epidermal growth factor (EGF) receptor family, which constitutes four members in mammals, ErbB-1/EGFR, ErbB-2/HER2, ErbB-3/HER3 and ErbB-4/HER4 [reviewed in (Yarden and Sliwkowski 2001)]. These receptors are activated by a large number of ligands, which belong to two major families—EGF-like ligands and neuregulins. The ligands direct formation of homo- or hetero- dimers of the receptors; ErbB-1-containing dimers are formed upon interaction with EGF-like ligands, while neuregulins direct formation of dimers containing ErbB-3 or ErbB-4. ErbB-2 appears to function as a ligand-less receptor (Klapper et al. 1999), whereas ErbB-3 has a defective kinase domain (Guy et al. 1994), such that it can signal only in the context of a heterodimer (Pinkas-Kramarski et al. 1996). The C-terminal tail of the four ErbB receptors is the most divergent domain among them, allowing for activation of distinct sets of effectors, which confers another level of complexity. Thus, the ErbB- family of RTKs forms a complex layered network of receptors and ligands (Alroy and Yarden 1997), where precise signaling can be achieved via action of a given ligand with a particular receptor pair, resulting in activation of a specific subset of signaling molecules. In line with the diversity of responses generated by this signaling network, down-regulation of each of the receptors appears to follow distinct pathways and kinetics, which are also varied in a manner dependent upon the identity of the stimulating ligand and the dimerization partner. In this manner, the major factor regulating termination of signaling by RTKs is also divergent among the different signaling units (Baulida et al. 1996; Levkowitz et al. 1996; Pinkas-Kramarski et al. 1996), allowing for an additional dimension of plasticity in signaling by this family of RTKs.

Two lines of research have been particularly useful for understanding signaling by the ErbB network. First, the mammalian network of ErbBs has

evolved from a well characterized signaling system in *C. elegans*, where a single receptor (LET-23) and a single ligand (LIN-3) are found. In insects, the single receptor (*Drosophila* EGF receptor, or DER) binds at least three stimulatory ligands and one antagonist (Perrimon and Perkins 1997). As will be emphasized in this review, many lessons can be taken from these systems, which are amiable to genetic manipulation and analysis. The second source of information is the interface with oncogenesis: The ErbB family of RTKs has been repeatedly implicated in cancer and other hyperproliferative disorders [reviewed in (Hynes and Stern 1994; Klapper et al. 2000a)]. Examples include autocrine loops involving ErbB ligands, and mutated versions of the receptors or their downstream effectors, which are encoded by pathogenic viruses, including retroviruses. Most relevant to human cancer is overexpression of ErbB-2 and ErbB-1, which occurs in different subsets of tumors. Due to the broad implication of these receptors in cancer, deep understanding of the mechanisms of their down-regulation is important, as these could serve as a point of intervention in cancer therapy.

Lessons from Endocytosis of Yeast Membrane Proteins

Yeast has proven itself as a reliable model system for research of cellular trafficking in eukaryotic cells (Munn 2001), because it appears that the principles underlying intracellular trafficking are shared from yeast to man. In addition, many of the proteins involved in trafficking events have conserved sequence, structure and function throughout eukaryotic evolution. Yeast confers the advantage of a fully sequenced, relatively simple genome, from which non-essential genes can be eliminated for direct examination of their role, and for which a large library of well characterized mutants, defective in many aspects of cellular physiology, is available. As the ubiquitin molecule and ubiquitination machinery are well conserved between yeast and mammals, seemingly also in terms of function, it appears that this system could also serve as a beacon for understanding the role and mechanism of ubiquitination in regulation of mammalian membrane proteins.

A number of yeast membrane proteins have been found to undergo ubiquitination at the cell surface. This ubiquitination serves a role in

marking the proteins for internalization, followed by vacuolar degradation [reviewed in (Hicke 1999; Hicke 2001)]. Ste2p, the yeast mating factor receptor, is a G-protein coupled receptor, which has been most intensively studied in this context. In response to its ligand, the receptor is rapidly phosphorylated, internalized and degraded in the vacuole. By contrast, in endocytosis-deficient cells Ste2p accumulates at the plasma membrane. Ubiquitination is necessary for rapid internalization of Ste2p, since mutants defective in ubiquitination of Ste2p exhibit a defect in Ste2p internalization (Hicke and Riezman 1996). On the other hand, ubiquitination of the receptor is sufficient to mediate the endocytic signal, as overexpression of a lysine-less ubiquitin, which is unable to nucleate poly-ubiquitin chains, does not affect internalization of Ste2p. Unlike many ubiquitinated yeast proteins, which are degraded by the proteasome, Ste2p is degraded in the vacuole, the counterpart of the lysosome of animal cells. In-frame fusion of ubiquitin to a truncated Ste2p lacking the remaining cytoplasmic tail lysine residues can restore Ste2p internalization (Terrell et al. 1998). In hand with these results, which assign to the ubiquitin molecule itself a function in signaling endocytosis in yeast, specific surface patches within ubiquitin act as signals for endocytosis (Shih et al. 2000). Interestingly, a possible function for poly-ubiquitin chains in signaling endocytosis has also been found in yeast: Apparently, endocytosis of the Fur4p uracil permease occurs already when a single ubiquitin is linked to the transporter. However, subsequent addition of ubiquitin molecules through lysine 63 of the first ubiquitin moiety is needed for normal rate of permease internalization (Galan and Haguenauer-Tsapis 1997).

Due to the close structural and functional relationships between yeast and mammalian endocytic processes, it is worthwhile to review the action of some of the major molecular players in endocytosis of yeast membrane proteins. These are the ubiquitin ligase Rsp5p (Nedd4 in mammals), Pan1p, the putative ortholog of Eps-15 of mammals, and Vps23p, whose ortholog is Tsg-101.

Rsp5p- Ubiquitination of several yeast membrane proteins, including Fur4p (Hein et al. 1995; Galan et al. 1996), have been found to be dependent on the HECT domain E3 ubiquitin-ligase Rsp5p. Both Nedd-4 and Itch of mammalian cells resemble the domain structure of Rsp5p. The protein is essential for cell viability in yeast, but mutations in Rsp5p are suppressed by

overexpression of ubiquitin (Zoladek et al. 1997). The Rsp5p/Nedd4 family of E3 ubiquitin ligases has in common the HECT domain, multiple proline-binding WW domains, and a calcium-dependent lipid-binding domain (CaL) [for a recent review see (Rotin et al. 2000)]. While this latter motif seems essential for association with the plasma membrane, the WW domains have been found to be essential for proper function of the protein. Recently, additional roles have been suggested for the ubiquitination activity of Rsp5p in regulating internalization, beyond ubiquitination of the cargo proteins themselves (Dunn and Hicke 2001). Both ubiquitin-dependent and independent endocytosis signals are apparently mediated by a Rsp5p-dependent ubiquitination event. In this context, a role has been proposed for Rsp5p in ubiquitination of a component of the constitutive endocytic machinery in yeast. A candidate target for the Rsp5p mediated ubiquitination is Pan1p, the yeast homologue of mammalian Eps15 (Zoladek et al. 1997; Wendland and Emr 1998). In line with the data for its yeast counterpart, Nedd-4 is involved in down-regulation of the mammalian epithelial sodium channel (ENaC), through direct binding of the WW domains of Nedd4 to proline-rich regions on ENaC (Staub et al. 1996; Staub et al. 1997). Internalization of ENaC is apparently dependent upon its ubiquitination by Nedd4. Moreover, mutations in ENaC, which abolish the interaction with Nedd4, have been associated with Liddle's syndrome, a hereditary form of systemic renal hypertension, due to an increase in the number of active channels at the plasma membrane and an associated increase in sodium influx. In this context, yeast has served as a reliable system, in which lessons learned as to the role of ubiquitination of plasma membrane proteins are applicable to mammals, and associated with a human disease.

Pan1p- Pan1p in yeast, and its mammalian homologue, EGFR protein substrate 15 (Eps15), are essential for normal endocytosis (Carbone et al. 1997; Benmerah et al. 1998; Wendland and Emr 1998). Although these proteins are associated with clathrin complexes and genetic evidence raised the possibility that Pan1p may act as an adaptor connecting Rsp5p to potential ubiquitination substrates, physical associations between Rsp5p and Pan1p have not been detected. In mammalian cells, Eps15 is tyrosine-phosphorylated and mono-ubiquitinated upon EGF stimulation (van Delft et al. 1997). The tyrosine-phosphorylation of the protein may play a role in

mediating accelerated endocytosis of ligand- activated ErbB-1 (Confalonieri et al. 2000). Expression of dominant-negative forms of Eps15 or microinjection of anti-Eps15 antibodies inhibited both ligand-dependent endocytosis of ErbB-1 and constitutive endocytosis of the transferrin receptor (Carbone et al. 1997; Benmerah et al. 1998), consistent with the role for Pan1p in yeast.

Vps23p- This vesicular protein and its mammalian homologue, Tsg101, function in late endosomal trafficking (Li et al. 1999; Babst et al. 2000). When mutated in mammalian cells, trafficking of the transferrin receptor remains normal, but down-regulation of the EGFR is attenuated. Analysis of the defect revealed that sorting of the receptor to multivesicular bodies is disrupted, leading to enhanced recycling to the cell surface. In yeast, mutant Vps23p permits recycling of damaged Ste2p molecules to the cell surface. Interestingly Vps23p and Tsg101 contain a domain similar to E2 ubiquitin-conjugating enzymes. However, this domain is unlikely to be active alone in catalysis of ubiquitination, as it lacks the active-site cysteine. However, a role may be hypothesized for this domain in recognition of ubiquitin. In this view, Tsg101/Vps23p may recognize ubiquitinated membrane proteins and direct them to multivesicular late endosomes (Lemmon and Traub 2000). Another possibility is that these proteins may recognize ubiquitinated proteins that are involved in trafficking of endocytic vesicles.

Ligand-Induced Ubiquitination and Endocytosis of Receptor Proteins

Conceivably, lessons learned in yeast on internalization of membrane proteins are relevant to both the constitutive and the inducible pathways of receptor endocytosis in mammals. The constitutive pathway is the least understood, and it may bypass a requirement for ubiquitination by utilizing intrinsic di-leucine and other signals for protein internalization (Govers et al. 1998). In the ErbB family, the pathway may be exemplified by the relatively slow internalization rate of mutant RTKs, either the kinase- defective ErbB-3 (Baulida et al. 1996; Baulida and Carpenter 1997; Waterman et al. 1998), or artificial kinase-defective mutants (Chen et al. 1989; Felder et al. 1990).

Apart from their slow rate of internalization, these receptors are only slightly affected by ligand binding and they are sorted to the recycling endosome rather than to the late endosome/pre-lysosome (Honegger et al. 1987; Hopkins et al. 1990). The more rapid and ligand-inducible route of internalization is covered by a number of extensive reviews (Di Fiore and Gill 1999; Carpenter 2000; Ceresa and Schmid 2000; Waterman and Yarden 2001). In this context, we shall focus on the role of ubiquitination in the ligand sensitive endocytic process. One mode of tuning of RTK signaling is via strict regulation of the breadth and time course of the receptor's active state (Moghal and Sternberg 1999; Fiorini et al. 2001). The major process regulating these characteristics of RTK signaling is endocytic removal of the active receptor from the cell surface. In recent years a view by which RTK signaling and endocytosis are intertwined is appearing; accordingly signaling from RTKs may occur along multiple points in the endocytic pathway. Consequently, particular signaling events occur only once the receptor has arrived at the endosomal compartment [reviewed in (Wiley and Burke 2001)], and the potential of a receptor to reach the endosome and activate signaling is regulated both by its dimerization partner and by the activating ligand [for an example see (Ebner and Derynck 1991)].

Upon ligand binding, an activated RTK is targeted to clathrin-coated membrane invaginations, which eventually pinch off to form clathrin-coated vesicles. Subsequent trafficking events deliver the contents of the vesicle through sequential endosomal compartments ending in receptor degradation. Various decision points occur on the path to receptor degradation, first among these is the endocytic step, followed by subsequent sorting to early endosomes, late endosomes and lysosomes. However, it seems that at each step the receptor can still be recycled back to the cell surface, although the efficiency of recycling is gradually decreased (Sorkin et al. 1991). Among the factors determining the fate of the receptor is the identity of the ligand, the co-receptor in the heterodimer, as well as the state of receptor serine/threonine and tyrosine phosphorylation. These in turn may effect coupling of the receptor to cellular proteins involved in the recognition of determinants important for receptor sorting. Examples can be seen with receptors of the ErbB family: ErbB-1 undergoes rapid endocytosis and degradation following ligand stimulation, but phosphorylation on a single threonine residue significantly affects receptor routing (Bao et al. 2000).

Unlike ErbB-1, endocytosis of ErbB-2 and the neuregulin receptors, ErbB-3 and ErbB-4, is relatively slow (Baulida et al. 1996; Pinkas-Kramarski et al. 1996). Additionally, as ErbB-3 is a kinase-defective receptor (Guy et al. 1994), it is sorted to recycling to the cell surface, after removal of the ligand in an endosomal compartment (Waterman et al. 1998). ErbB-2 has a negative effect on ligand-mediated endocytosis of ErbB-1, causing prolonged retention of the receptor in the vicinity of the plasma membrane, as well as enhanced recycling back to the cell surface (Sorkin et al. 1993; Lenferink et al. 1998; Worthylake et al. 1999). As a result, ErbB-2 extends retention of its heterodimer partners at the cell surface, thereby prolonging signaling. Because overexpression of ErbB-2 biases formation of heterodimers over homodimers, overexpression of ErbB-2 in human malignancies has a significant effect on prolonging ligand-mediated signaling, and consequently enhances mitogenicity of ErbB ligands.

Early Reports on Ligand-Induced Ubiquitination of RTKs

A highly important role is attached to ubiquitination in regulating ligand-induced endocytosis and degradation of membrane receptors. The first hints for ubiquitination of plasma membrane proteins appeared in the mid-1980s, when N-terminal sequencing of an isolated receptor for the platelet-derived growth factor (PDGF) yielded the sequence of ubiquitin (Yarden et al. 1986). Ligand-dependent ubiquitination of surface receptors was then detected with the T-cell receptor, which was found to be ubiquitinated on multiple lysine residues (Cenciarelli et al. 1992), including lysines introduced in positions where they did not normally exist (Hou et al. 1994). Subsequently, the PDGF receptor (Mori et al. 1992; Mori et al. 1993), as well as the EGF receptor (Galcheva-Gargova et al. 1995) and the stem cell factor receptor (c-Kit) (Miyazawa et al. 1994) were reported to undergo ligand-induced ubiquitination. A link between tyrosine kinase activity and receptor ubiquitination was suggested, as abolishing intrinsic kinase activity in RTKs caused a reduction in ubiquitination of the receptors. Inhibition of tyrosine phosphatase activity with resultant enhancement of receptor tyrosine phosphorylation was correlated with increased ubiquitination of the T-cell receptor (Cenciarelli et al. 1992). It is important to note that in contrast to the common role for ubiquitination in targeting proteins to

degradation by the proteasome (Hershko and Ciechanover 1998), degradation of poly-ubiquitinated RTKs was inhibited only in part by inhibitors of proteasomal proteinases (Mori et al. 1995a; Mori et al. 1995b; Jeffers et al. 1997). However, inhibitors of lysosomal enzymes were more effective, consistent with a role for ubiquitination in targeting RTKs to lysosomal destruction, rather than to proteasomal degradation.

Lines of Evidence Supporting a Role for Cbl in Negative Regulation of RTKs

The first hints for the molecular basis of the ligand-induced down-regulation and ubiquitination of RTKs came from genetic analyses of mutant worms. Certain loss of function mutations of LET-23, the worm homologue of the EGFR, could be suppressed by a mutant of the *sli-1* gene, consistent with disabling an inhibitory pathway (Jongeward et al. 1995; Yoon et al. 1995). The mutation affected the SH2 domain of SLI-1, the worm homologue of the mammalian c-Cbl proto-oncoprotein (Langdon et al. 1989b), and thereby suppressed a vulvaless phenotype of worms. Studies in *Drosophila* also suggested that Cbl proteins negatively regulate RTK signaling, as D-Cbl can suppress R7 photoreceptor development on a sensitized genetic background (Meisner et al. 1997), and it functions as a negative regulator of a dose-sensitive EGFR pathway involved in oogenesis (Pai et al. 2000). Evidence for a similar role for the mammalian c-Cbl came from reports that it can attenuate signaling from a number of cellular receptors, such as the IgE receptor (Ota and Samelson 1997), the T-cell receptor (Rellahan et al. 1997), the B-cell receptor (Yankee et al. 1999), the EGF receptor (Levkowitz et al. 1998), the PDGF receptor (Miyake et al. 1998; Miyake et al. 1999) and the colony-stimulating factor 1 (CSF-1) receptor (Lee et al. 1999). In line with these in vitro observations, targeted disruption of the *c-cbl* gene led to increased cellularity in the thymus, spleen and lymph node (Murphy et al. 1998). Macrophages derived from c-Cbl$^{-/-}$ mice demonstrated enhanced proliferation in the presence of CSF-1 compared to normal mice (Lee et al. 1999). Consistent with a role for Cbl in negative regulation of LET-23/EGFR signaling, c-Cbl$^{-/-}$ mice exhibited hyperplasia of the mammary duct, a process regulated by EGF and TGF-alpha (Xie et al. 1997; Rudland et al. 1998). Disruption of a c-Cbl homologue, Cbl-b, in mice

displayed similar phenomena, in which peripheral T-cells were uncoupled from a requirement for activation of the co-receptor CD28. Consequently, Cbl-b$^{-/-}$ mice are highly susceptible to an autoimmune disease (Bachmaier et al. 2000; Chiang et al. 2000).

Another important line of evidence supporting a negative role for c-Cbl came from analyses of natural and engineered oncogenic mutants of the protein. The c-Cbl proto-oncogene is the cellular homologue of v-Cbl, the gene responsible for the transforming ability of the Cas NS-1 retrovirus, a murine virus that causes pre-B lymphoma and myeloid leukemia (Langdon et al. 1989a). Four oncogenic mutants of the protein have been identified, namely: v-Cbl, a C-terminally truncated form identified in a transforming retrovirus (Blake et al. 1991), 70Z-Cbl, which was isolated from the 70Z/3 pre-B cell lymphoma, in which there is an internal deletion of 17 amino acids (Blake et al. 1991), and delta-Y368- and delta-Y371-Cbl, in which specific tyrosine residues of the protein are deleted (Andoniou et al. 1994). Interestingly, the oncogenic mutations of c-Cbl concentrate around the centrally located RING finger, which has recently been identified as an association site for E2 ubiquitin conjugating enzymes [reviewed in (Jackson et al. 2000; Joazeiro and Weissman 2000)]. The RING finger of c-Cbl is of the C3HC4 type containing one histidine in a metal coordination position. By contrast, the RING-H2 fingers found in multi-protein ubiquitin ligases, such as Rbx, Roc1, and APC11, contains two histidines in the metal coordination positions. The RING is a zinc-coordinating domain, which organizes in two zinc binding loops. The zinc-coordinating residues are essential for RING function, which is most likely binding to E2 ubiquitin-conjugating enzymes, and promoting polymerization of poly-ubiquitin chains. The recently reported crystal structure of c-Cbl (Meng et al. 1999; Zheng et al. 2000b) suggests that the function of the RING finger is to act as a scaffold, bringing the substrate into close proximity with the E2 enzyme, so that the transfer of ubiquitin may occur.

The Function of c-Cbl in Down-Regulation of Ligand-Activated RTK

Early into the investigation of the mechanism of action of c-Cbl, this protein was found to undergo phosphorylation upon activation of cell surface receptors, and to associate with the receptors (Bowtell and Langdon 1995;

Galisteo et al. 1995; Tanaka et al. 1995). However, coupling to c-Cbl is not common to all RTKs. Within the ErbB family, c-Cbl is found associated with ErbB-1/EGFR upon stimulation, but only weakly with ErbB-2, and no coupling of Cbl to ErbB-3 or ErbB-4 has been reported (Levkowitz et al. 1996; Waterman et al. 1999a; Klapper et al. 2000b; Levkowitz et al. 2000). This differential recruitment of c-Cbl to ErbB receptors is inversely correlated with the ability of these receptors to undergo ligand-induced down-regulation (Baulida et al. 1996; Pinkas-Kramarski et al. 1996; Levkowitz et al. 1998; Muthuswamy et al. 1999), hinting to a role for c-Cbl in the down-regulation of RTKs. Indeed, Cbl overexpression has been found to enhance ubiquitination and down-regulation of the PDGFR (Miyake et al. 1998), the EGFR (Levkowitz et al. 1998) and the CSF-1 receptor (Lee et al. 1999). Moreover, v-Cbl and 70Z-Cbl, two oncogenic forms of Cbl, have been found to interfere with ligand-induced down-regulation of these receptors, and the activity depends on an intact RING finger of c-Cbl (Waterman et al. 1999b). The mechanism through which c-Cbl induces down-regulation was resolved by use of a cell-free in-vitro assay. This led to identification of the E3 ubiquitin-ligase activity of the protein, involving the RING domain in binding of E2 enzymes (Joazeiro et al. 1999; Levkowitz et al. 1999; Yokouchi et al. 1999). The activity of c-Cbl appears tightly regulated by two tyrosine phosphorylation events (Levkowitz et al. 1999), the first event is autophosphorylation of the receptor (tyrosine 1045 in the EGFR), creating a docking site for the unique SH2 domain of c-Cbl. The second event has been inferred on the basis of mutagenesis and awaits direct proof of phosphorylation at the identified site (Levkowitz et al. 1999). Accordingly, phosphorylation of c-Cbl on a tyrosine residue within the linker domain between the TKB and the RING finger (tyrosine 371), activates Cbl's ubiquitin-ligase activity. The intact amino-terminal part of the protein, including the SH2 and RING domains, is necessary and sufficient for ubiquitination and down-regulation of the receptor, and the activity of c-Cbl was even found to be enhanced upon truncation of the C-terminal part (Levkowitz et al. 1999; Lill et al. 2000). As the RING-finger of c-Cbl and the proximal linker-domain are missing or mutated in the oncogenic forms of the protein, it has been suggested that the mechanism of transformation by v-Cbl and 70Z-Cbl is through disruption of c-Cbl-mediated ubiquitination of its substrates. Indeed, co-expression of v-Cbl

with c-Cbl counteracted the down regulatory activity of the wild type protein and also enhanced recycling of EGFR back to the cell surface (Levkowitz et al. 1998).

The sub-cellular location of c-Cbl's action is still an open question that reflects on the exact function in receptor ubiquitination. c-Cbl was found in endosomes (Meisner and Czech 1995; Tanaka et al. 1995), where it was co-localized with ligand stimulated EGFRs (Levkowitz et al. 1998). In addition, the c-Cbl binding site on the EGFR (tyrosine 1045), is embedded in a region previously mapped to a lysosomal target motif (Kornilova et al. 1996), and Cbl proteins defective in ubiquitination enhance recycling of the EGFR (Levkowitz et al. 1999; Waterman et al. 1999b). In addition, overexpression of c-Cbl in cultured fibroblasts dramatically enhanced ubiquitination of EGFR, but exerted no effect on the rate of ligand endocytosis (Levkowitz et al. 1998; Thien et al. 2001). On the other hand, c-Cbl has been reported to associate with the CSF-1 receptor of macrophages already at the cell membrane (Lee et al. 1999; Wang et al. 1999). Also in support of the possibility that c-Cbl affects receptor ubiquitination prior to internalization are experiments performed with cells overexpressing a dominant-negative form of dynamin (Stang et al. 2000). A clear answer to the question of sub-cellular localization of c-Cbl's action has emerged from our recent analyses of an EGFR mutated at the c-Cbl's docking site (Y1045F). Ligand-induced internalization of EGFR was significantly retarded by defecting the interaction with c-Cbl. In addition, the rates of receptor down regulation and ligand degradation were inhibited almost to the level exhibited by a kinase-defective mutant receptor (Waterman et al. 2001). Likewise, the rate of ligand recycling by Y1045F was comparable to that of the kinase-defective receptor, whose sorting to late endosomes is known to be defective (Felder et al. 1990; Hopkins et al. 1990). The internalization-defective Y1045F mutant not only remained at the cell surface after stimulation with EGF, but it could not mediate translocation of c-Cbl into endosomes. It is therefore conceivable that ubiquitination by c-Cbl occurs already at the level of the plasma membrane and it determines the rate of receptor endocytosis by enhancing sorting to the invaginating clathrin coated pit. The schematic model presented in Figure 1 summarizes the current view of c-Cbl action in RTK endocytosis.

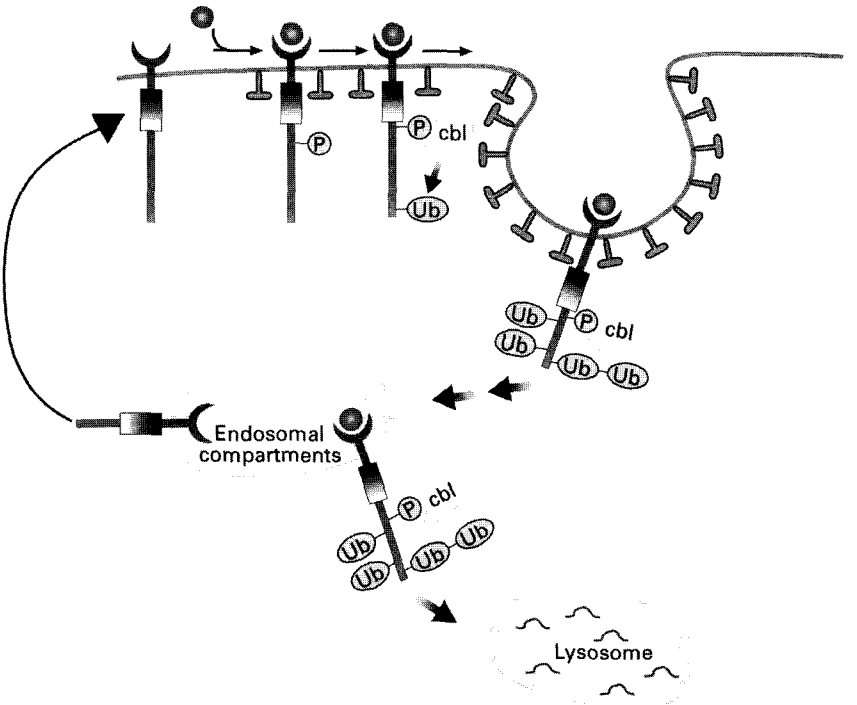

Figure 1. Proposed mechanism of ligand-induced RTK endocytosis. The model is based upon results obtained with EGFR (see text for references). Ligand binding to a surface-localized receptor promotes autophosphorylation of several C-terminally located tyrosine residues, including a c-Cbl-specific tyrosine based docking site (tyrosine 1045 in EGFR). Presumably, only a small fraction of cellular c-Cbl molecules translocate from cytoplasmic locations to the activated, membrane-bound receptor. Consequent to binding of c-Cbl, an associated ubiquitin-loaded E2 molecule transfers ubiquitin to the receptor. It is currently unknown which sites on the receptor are ubiquitinated and what is the type of ubiquitination (mono- or poly-ubiquitination). However, it seems that the attachment of ubiquitin allows recruitment of the tagged receptor to the clathrin coated pit (clathrin is represented by T shaped structures). Cbl molecules appear to escort the internalizing receptor along the endocytic pathway, perhaps in order to sequentially add more residues which may be needed for additional vesicular sorting events. Eventually, the poly-ubiquitinated receptor is targeted to late endosomal compartments where lysosomal hydrolases degrade the ligand-receptor complexes. However, in case de-ubiquitination takes place or non-ubiquitinated receptors are somehow internalized, they are destined for recycling back to the cell surface by an unknown mechanism.

Two Alternative Pathways of c-Cbl Recruitment and Ubiquitination of RTKs

The structure of c-Cbl consists of an N-terminal tyrosine kinase binding domain (TKB), which is structured from a four-helix bundle, an EF-hand and a variant SH2 domain (Meng et al. 1999). Following the TKB domain is a short alpha-helical linker domain, followed by the RING finger. The C-terminal half of the protein construes an acidic region, followed by a proline rich region, allowing for up to 15 independent SH3 binding sites, intertwined with multiple tyrosine phosphorylation sites. The very carboxy terminal tail of the protein comprises of an ubiquitin binding domain (UBA) and a leucine zipper. Consistent with a regulatory role for the region distal to the RING finger, c-Cbl is a prominent phosphoprotein, which has been found to undergo tyrosine phosphorylation in response to various extracellular stimuli, including cytokines, antigens and growth factors [reviewed in (Lupher et al. 1998)]. c-Cbl phosphorylation on tyrosine residues localized to the carboxyl terminal half is mediated by Src family members (Feshchenko et al. 1998) and it establishes binding sites for several SH2-containing adaptors and enzymes (e.g., PI3K, Vav and Crk) [for a recent review see (Thien and Langdon 2001)]. Unlike this inducible type of interactions, the poly-proline region of c-Cbl mediates constitutive binding of several SH3-containing proteins, of which Grb2 is the most extensively characterized.

Similar to c-Cbl, the Grb2 adaptor protein is involved in many signal transduction pathways, including those initiated by growth factors, antigens and antibodies [reviewed in (Clements et al. 1999)]. Its single SH2 domain allows inducible binding to tyrosine-phosphorylated proteins, whereas the two flanking SH3 domains recruit signaling proteins, including the guanine nucleotide exchange protein Sos (Lowenstein et al. 1992; Buday and Downward 1993) and the E3 ubiquitin ligase, c-Cbl (Donovan et al. 1994; Fukazawa et al. 1995; Meisner et al. 1995). The interaction with Sos is relatively well characterized; it recruits the exchange protein to the plasma membrane and activates the Ras pathway. Although a complex of c-Cbl and Grb2 is abundant in many cell types and its dissociation may be regulated (Buday et al. 1996; Donovan et al. 1996), the functional consequences of

recruiting a c-Cbl Grb2 complex to activated receptors was unknown until very recently.

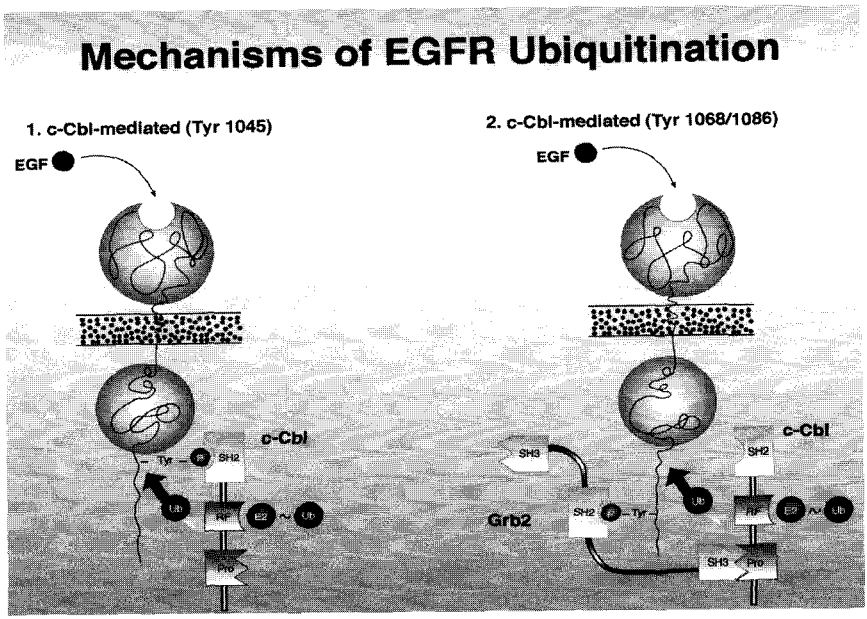

Figure 2. The alternative pathways of EGFR ubiquitination by c-Cbl. The EGFR is shown as a bilobular structure that crosses the plasma membrane (stippled). Mechanism 1 involves direct recruitment of c-Cbl to EGFR through binding of the N-terminally located SH2 domain of c-Cbl to a phosphorylated tyrosine at position 1045 distal to the globular kinase domain. This pathway seems to be the predominant route of ligand-induced ubiquitination of EGFR in living fibroblasts. Similarly, the alternative mechanism is initiated upon ligand binding and stimulation of receptor auto-phosphorylation. However, recruitment of c-Cbl to the phosphorylated receptor is mediated by tyrosine residues other than tyrosine 1045. For example, tyrosine residues 1068 and 1086 are potential docking sites for Grb2. Presumably, the adaptor protein, through one of its SH3 domains, is pre-complexed to the c-Cbl's proline-rich domain (*Pro*). It is proposed that the ternary complex comprising EGFR, Grb2, c-Cbl, and an associated ubiquitin-loaded E2 enzyme can ubiquitinate the receptor. Currently it is unknown whether the two mechanisms differ in aspects related to sub-cellular localization and association with other molecules.

Analysis of a mutant EGFR incapable of direct interaction with c-Cbl (Y1045F mutant) revealed some residual ligand-induced and c-Cbl-dependent ubiquitination of the point mutated variant of EGFR. By screening a series of c-Cbl mutants we concluded that the proline-rich domain of c-Cbl was essential for weak Y1045F ubiquitination by EGF. In line with the possibility that EGF binding recruits a complex of Grb2 and c-Cbl, overexpression of Grb2 enhanced ligand-induced ubiquitination, internalization and degradation of the mutant receptor (Waterman et al. 2001). Thus, at least two distinct mechanisms allow inducible ubiquitination and degradation of EGFR (Figure 2): The major one is mediated by tyrosine 1045, which directly binds to the SH2 domain of c-Cbl (Levkowitz et al. 1999). The secondary mechanism seems to involve one of the Grb2 binding sites on EGFR (tyrosine autophosphorylation sites 1068, 1086 and presumably additional residues), and indirect recruitment of c-Cbl through its constitutive interactions with an SH3 domain of the adaptor. Our experiments detected no functional differences between the two mechanisms besides the dominance of the tyrosine 1045-mediated mechanism. However, it is possible that differential kinetics of modification of individual autophosphorylation sites (Batzer et al. 1994), or intracellular compartmentalization of Grb2 (Sorkin et al. 2000), can temporally or spatially control the two mechanisms of receptor inactivation.

The involvement of Grb2 in endocytosis is consistent with two previous lines of evidence. First, microinjection of a recombinant SH2 domain of Grb2 inhibited endocytosis of EGFR in living cells (Wang and Moran 1996). Because the corresponding domains of other EGFR-interacting proteins, like the Ras GTPase-activating protein, the SH2 and collagen homology protein (SHC) and PI3K were inactive, it seems likely that the effect of Grb2 is unique. Second, deletion of the Grb2-binding poly-proline motif of SLI-1 significantly affected vulva formation in a transgenic functional assay established in worms (Yoon et al. 2000). Together with the effect of Grb2 on c-Cbl-induced ubiquitination, these lines of evidence attribute to the adaptor a dual role in signaling; along with the extensively characterized stimulatory activity of the Ras pathway through binding to SOS, Grb2 also initiates the process of receptor degradation by recruiting c-Cbl. Because c-Cbl and SOS do not co-immunoprecipitate (Meisner et al.

1995; Fukazawa et al. 1996), they seem to form exclusive complexes with Grb2. Thus, it is conceivable that by interacting with SOS and c-Cbl, Grb2 integrates both positive and negative inputs to signaling pathways. For example, a Grb2 Cbl complex may replace a cell surface-associated complex of Grb2 and SOS. The latter is engaged in Ras activation by EGFR, but it may fall apart when the receptor enters the endocytic route. Indeed, Grb2 seems to interact with EGFR in the membrane, as well as in endosomal compartments (Sorkin et al. 2000). In addition, although the interaction of Grb2 and c-Cbl is mediated by constitutive binding, according to some reports the complex may dissociate in response to receptor activation (Buday et al. 1996; Donovan et al. 1996). The underlying mechanism is still unknown, but it could be part of a regulatory cycle of Ras activation and inactivation by Grb2. This type of control may be especially important in the case of receptors that cannot recruit c-Cbl in a direct way and must rely on Grb2 for their ubiquitination. However, other adaptor proteins may also compensate for the absence of a c-Cbl-specific phosphotyrosine-docking site. One such example is the APS adaptor, which has been reported to couple the insulin receptor to c-Cbl, and to facilitate ligand-dependent receptor ubiquitination (Ahmed et al. 2000).

Open Questions

Recently a role was assigned for de-ubiquitination in activating endocytosis of membrane proteins in *Drosophila* retinal progenitor cells. The de-ubiquitinating enzyme, Fat facets (Faf, a UBP enzyme), interacts with Lqf, a homologue of the mammalian protein epsin, which is involved in the endocytic pathway (Cadavid et al. 2000). Deubiquitination of Lqf apparently activates its endocytic function, probably accelerating endocytosis of a yet uncharacterized membrane protein. Whether de-ubiquitination of the endocytic machinery can indeed accelerate its function is currently an open question. By contrast with this notion, endocytosis of the growth hormone receptor depends on an intact ubiquitination machinery and a short receptor's cytoplasmic domain (Govers et al. 1999). However, because no lysine residue of the cytoplasmic portion is necessary for receptor internalization, it seems that ubiquitination of a component of the endocytic machinery, rather than the receptor itself is required for effective

endocytosis of the growth hormone receptor. Lastly, it is currently unknown whether mono-ubiquitination, as opposed to poly-ubiquitination of membrane receptors, is sufficient for their internalization. Mono-ubiquitination could occur on several lysine residues of an internalizing RTK, or ubiquitination could take place on a single lysine and proceeds by ubiquitin branching. These two modes of RTK modification are difficult to resolve by biochemical methods when analyzing ubiquitination in intact cells.

Stress-Induced Ubiquitination and Degradation of Receptor Tyrosine Kinases

Biological systems are subject to a high level of regulation by proofreading. At the protein level, this function is served by the cellular chaperones, which monitor the fold of the protein, acting to refold proteins to their native structure. In recent years a slightly modified view of the function of chaperones is emerging. Accordingly, apart from their role during protein synthesis, the chaperones serve as part of a 'triage' system in the cell. This system is highly sensitive to the three dimensional structure of cellular proteins. The model proposes that a non-native protein be subjected to rounds of attempted refolding. If this refolding is unsuccessful, the protein is directed to a degradative fate, utilizing the ubiquitin conjugation system and the proteasome. Interestingly, some signaling proteins such as steroid hormones and protein kinases, are monitored by chaperones (Buchner 1999), but coupling to receptor tyrosine kinases is limited to a subgroup that contains ErbB-2 and the receptors for insulin and the hepatocyte growth factor (Sepp-Lorenzino et al. 1995; Chavany et al. 1996; Stancato et al. 1997; Sakagami et al. 1999; Tikhomirov and Carpenter 2000; Webb et al. 2000).

Chaperone Interactions with Receptor Tyrosine Kinases

The model of protein triage has been presented by Gottesman and colleagues (Gottesman et al. 1997; Wickner et al. 1999). Protein triage is suggested to occur through a dynamic, ATP-dependent interaction of non-

native polypeptides with chaperones. This interaction is limited; the majority of cellular proteins does not interact with chaperones, and are resistant to the degradative machinery due to the intrinsic stability of their structures. Continuous chaperone association and dissociation events lead to refolding of the polypeptide to its native conformation, such that it leaves the pool of proteins amenable to refolding attempts. Alternatively, it may lead to the recognition that the protein cannot be effectively refolded, and its subsequent degradation. Gottesman and colleagues (Wickner et al. 1999) suggested that the critical partitioning step between refolding and degradation will be a competition between chaperones and components of the ubiquitin system, as protein ubiquitination mediates recognition by the proteasome. Our work on receptor tyrosine kinases lead us to suggest a slightly modified view of the eukaryotic triage system, in which the chaperones may actively act to direct unfolded proteins to ubiquitination. In this manner, we suggest that association of non-native proteins with chaperones may lead to their presentation to the ubiquitin machinery in a yet undefined manner.

In mammalian cells, the triage system appears to comprise a multi-molecular complex of a number of chaperones and co-chaperones, with which the proteasome and the ubiquitinating machinery interact. Shuffling of chaperone complexes seems to play an active role in ubiquitination of the chaperoned proteins and their subsequent degradation. The core of the chaperoning triage complex consists of the Hsp90 family of chaperones. Several protein kinases appear to be regulated by the Hsp90-based triage system. This growing list includes members of the Src, Fak and Abl families of tyrosine kinases, cyclin-dependent kinase Cdk4, and another cell cycle associated kinase-Wee1, as well as the Raf1 serine/threonine kinase and several RTKs. Association of receptor tyrosine kinases with this chaperone can regulate their activity at two cellular junctions, the first is protein maturation through the endoplasmic reticulum (Murakami et al. 1994; Supino-Rosin et al. 2000), and the second is the state of signaling competence of the mature protein at the plasma membrane (Neckers et al. 1999a).

A significant breakthrough in identifying protein clients of Hsp90 was the realization that the chaperone is inhibited by benzoquinone ansamycins like geldanamycin and Herbimycin A (Whitesell et al. 1994). The first

identified RTK client of Hsp90 was ErbB-2 (Miller et al. 1994a; Miller et al. 1994b), which was found to be susceptible to herbimycin A-induced degradation. The region of susceptibility mapped to amino acids 751-957, corresponding to the kinase domain of the receptor. The ability of benzoquinoid ansamycins to cause degradation of ErbB-2 was initially correlated with binding of the compounds to Grp94, an Hsp90 family member primarily localized to the endoplasmic reticulum (Chavany et al. 1996). Later studies, however, indicated that ErbB-2 degradation is affected by inactivation of Hsp90, rather than Grp94, by the ansamycin drugs (Xu et al. 2001). Thus, alterations in ErbB-2/chaperone interactions leads to poly-ubiquitination of the receptor, endocytosis, and subsequent degradation by the proteasome. Evidence for this model has been provided by the detection of ErbB-2 poly-ubiquitination that precedes degradation and the ability of proteasome inhibitors to retard proteolysis of ErbB-2 following treatment of cells with geldanamycin. The ansamycin drugs were later found to interfere with the maturation of growth factor receptors, causing proteasomal degradation of the nascent forms of ErbB-1 and PDGFR, and not affecting the mature forms of these receptors (Sakagami et al. 1999). Insulin receptor and IGF1 receptor have also been found to undergo herbimycin A-induced degradation and this was dependent on an active E1 ubiquitin activating enzyme (Sepp-Lorenzino et al. 1995). Later studies confirmed physical association between Hsp90 and the insulin receptor (Takata et al. 1997). The Met receptor probably serves as another client of Hsp90 as its degradation is retarded by inhibitors of proteasomal proteinases and its stability is sensitive to GA (Webb et al. 2000).

Both mature and nascent forms of ErbB-2 are sensitive to geldanamycin. The drug seems to prevent exit of nascent ErbB-2 from the endoplasmic reticulum (Chavany et al. 1996), and directs the precursor to degradation by the proteasome (Mimnaugh et al. 1996). Sensitivity of ErbB-2 is conferred by the kinase domain of the receptor, but the functionality of the kinase is not necessary, as a kinase-defective mutant of ErbB-2 is also sensitive to geldanamycin (Xu et al. 2001). ErbB-2 is subject to a complex form of degradation, involving the sequential activity of a caspase family protease that cleaves the carboxyl-terminal tail of the receptor from the rest of the molecule (Tikhomirov and Carpenter 2000; Tikhomirov and Carpenter 2001). The cytoplasmic part is ubiquitinated and is subjected to

proteasomal degradation, while the membranal fragment may be destined for lysosomal degradation.

Whereas the two neuregulin receptors, namely ErbB-3 and ErbB-4, seems uncoupled to Hsp90 (our unpublished observations), the immature form of ErbB-1 serves as a client of the triage system. For example, Herbimycin A accelerates degradation and prevents complete glycosylation of EGF-receptor in human tumor cells (Murakami et al. 1994). Because the receptor is sensitive to endoglycosidase H, it seems that only the precursor form is sensitive to the drug. These results demonstrate that RTKs exhibiting sensitivity to anasamycins can be divided into two categories: those that are susceptible to degradation of the mature form, like ErbB-2, and receptors in which only the nascent form is sensitive to geldanamycin. The sensitivity of a protein to geldanamycin usually reflects chaperoning by Hsp90. Hence, while some RTKs need association with Hsp90 for proper maturation, and therefore are sensitive to the drug at this stage, a select few RTKs are chaperoned by Hsp90 also as mature proteins, exhibiting geldanamycin sensitivity at the level of the membrane. Interestingly, the differential sensitivity of the mature forms of ErbB-1 and ErbB-2 correlates with differential susceptibility of the proteins to heat shock, as ErbB-2 displays much higher susceptibility to heat shock than does ErbB-1 (Liu and Carpenter 1993).

Mechanisms of Chaperone-Associated Ubiquitination and Degradation of Receptor Tyrosine Kinases

The mechanisms of action of molecular chaperones in the context of RTK degradation have proved to be relatively complex. Unlike the ligand-induced pathway of ubiquitination and degradation of RTKs, complete understanding of chaperone-mediated degradation of receptors and other clients is still lacking. The best-studied clients of Hsp90 are steroid hormone receptors for which a satisfactory model has been worked out [for a recent review and references see (Buchner 1999)]. Accordingly, the promiscuous chaperone interacts with client proteins at various stages of their maturation to help refold them or, alternatively, to direct their destruction. An intermediate complex containing both Hsp70 and Hsp90, along with the co-

chaperones HOP, Hip and Hsp40, undergoes maturation when Hsp70 dissociates (along with its co-chaperones) and two new co-chaperones join the complex: p23 and one of the large immunophilins. This complex maintains steroid hormone receptors in an inactive, but activatable state. Hormone binding to the receptor triggers dissociation from Hsp90 and subsequent binding of the hormone-bound receptor to specific elements of DNA. Alternatively, the dissociated complex may recruit Hsp70 and start another cycle. By inhibiting the ATP-dependent binding of p23 to Hsp90, geldanamycin prevents transition from an intermediate complex to the mature, signaling competent state. Thus, geldanamycin-induced destabilization of its clients may be secondary to stabilization of complexes containing Hsp70, in addition to Hsp90 and p60-HOP. Indeed, the heat shock cognate protein Hsc70, is involved in degradation of a subset of cellular proteins, probably through binding to hydrophobic patches exposed on the surface of unfolded client proteins, and promoting their ubiquitination and proteasomal degradation (Bercovich et al. 1997). Although detailed analyses of the association of RTKs with heat shock proteins or their cognate chaperones are still lacking, recent studies of three oncogenic tyrosine kinases, ErbB-2, v-Src and the p210 fusion protein (BCR-ABL), suggest that these kinases are associated with Hsp90 and its co-chaperone, p23. However, exposure of cells to geldanamycin replaces the co-chaperones with p60-HOP and Hsp70, prior to degradation of the client kinases (An et al. 2000; Xu et al. 2001). It is interesting that c-Src and c-Abl, the normal homologues of v-Src and p210 are less susceptible to geldanamycin. Similarly, ErbB-2, whose oncogenic potential exceeds that of ErbB-1, is complexed with Hsp90 but the mature form of ErbB-1/EGFR seems uncoupled to the chaperone.

Because the details of the chaperone-RTK cycle are still unclear, it is worthwhile reviewing the data currently available on candidate players of the cycle. Besides the two chaperones, Hsp90 and Hsp70, p50-CDC37 and the recently identified ubiquitin ligase, CHIP, are likely involved in degradation of RTKs through the chaperone pathway.

Hsp90

Hsp90 is one of the most abundant proteins in eukaryotic cells, comprising 1-2% of total cellular protein, even under non-stress conditions. This molecular chaperone is highly conserved throughout evolution, with homologues in bacteria and fungi. In contrast with its high abundance, Hsp90 participates in multiple yet restricted signal transduction pathways. Hsp90, in its function as a chaperone, is characterized by low affinity interactions with its substrates, and repeated cycles of binding and release (Smith et al. 1998). In this manner, Hsp90 keeps its inherently unstable client proteins poised for activation until they are stabilized by conformational changes induced by stimulation. Hsp90 recognizes structural features common to unstable proteins, such as unveiled hydrophobic patches, rather than specific sequence motifs. As minor changes in amino-acid sequences can have substantial effects on the conformational stability of a protein, large variability can be seen in the dependence of individual members of very homologous protein families on the interaction with Hsp90. Examples can be seen in the family of steroid-hormone receptors, the group of cyclin-dependent kinases (Stepanova et al. 1996), Src family kinases (Xu and Lindquist 1993; Bijlmakers and Marsh 2000), and the ErbB family of RTKs (Xu et al. 2001). Soluble kinases of the Src family, namely Lck, c-Src and Lyn, require association with Hsp90 for proper synthesis and membrane association, but not for maintenance. Therefore, these kinases associate with the chaperone until reaching the membrane, upon which they dissociate from Hsp90. However, once mutated to a constitutively active form (through mutations outside the kinase domain) Lck requires constitutive Hsp90 chaperoning (Bijlmakers and Marsh 2000). In an analogous way, the significance of the interaction of Hsp90 with ErbB-2, but not with other members of the family, may be related to the observation that this kinase exhibits relatively high tyrosine kinase activity, even in the absence of a stimulating ligand (Lonardo et al. 1990).

Hsp70

The Hsp70 family of chaperones is highly abundant and found in diverse cellular compartments. The unifying action in Hsp70 function is the ATP-

dependent binding to short hydrophobic regions of 6-9 amino acids in extended conformation [reviewed in (Mayer and Bukau 1998)]. As previously discussed, Hsp70, together with Hsp60 and Hsp90, comprise the minimal complex necessary and sufficient for folding steroid hormone receptors into a ligand-binding conformation. Various reports implicate Hsp70 in relation to ubiquitination and degradation of proteins in the cell: Overexpression of Hsp70 has been found to enhance ubiquitination and degradation of ApoB in the endoplasmic reticulum through the proteasome (Fisher et al. 1997). In yeast, mutation of Ydj1, a Hsp70 homologue, caused a large defect in ubiquitination and degradation of specific ubiquitin-proteasome substrates, such as Gcn4, with no effect on lysosomal substrates (Lee et al. 1996). Ydj1 was also shown to form complexes with these substrates. An additional Hsp70 homologue in yeast, SSB1, has been found to suppress defects of mutations in the proteasome (Ohba 1994). The Hsp70 family member, Hsc70, is needed for ubiquitination and degradation of some model proteins in vitro (Bercovich et al. 1997). Similar to the case of Hsp90, the chaperone and the degradation substrate form physical associations, but participation of co-chaperones was not investigated.

p50^{CDC37}

CDC37 is regarded as a link between Hsp90 and a specific subset of client proteins, namely, protein kinases. The adaptor is found in a host of Hsp90 kinase complexes, including those containing v-Src and Raf-1. Initial studies identified Cdc37 in mutant yeast cells arrested in G_1 (Reed 1980). In addition, an insect homologue was identified as a positive regulator of the *Sevenless* pathway, which culminates in MAPK activation (Cutforth and Rubin 1994). Subsequent studies suggested that the phenotype of *cdc37* is due to the interaction of Cdk4 with Hsp90 and p50-CDC37. Thus, the adaptor functions in both cell cycle regulation and the MAPK pathway. As is the case with Hsp90, CDC37 demonstrates a high level of specificity for its substrates, interacting with particular members of kinase families. For example, CDC37 has been shown to interact in a specific manner with Cdk4, excluding interactions with other members of the Cdk family such as CDK2, 3 and 5 (Dai et al. 1996). Likewise, CDC37 binds directly to the

kinase domain of Raf-1, and links the kinase to Hsp90 (Silverstein et al. 1998). Although Hsp90 can bind directly to Raf-1, p50 seems to stabilize a ternary kinase-adaptor-chaperone complex, which stimulates kinase activity (Grammatikakis et al. 1999). Another demonstration of the stimulatory effect of CDC37 is provided by the observation that a temperature- sensitive kinase defective mutant of Zap-70, which is constitutively degraded, is stabilized by overexpression of CDC37 (Matsuda et al., 1999). Consistent with its positive effect on MAPK signaling, p50-CDC37 can transform cells and it acts as an oncogene in mice (Stepanova et al. 2000). These lines of biochemical and genetic evidence support the possibility that similar to the effect of CDC37 on the serine/threonine kinase Raf-1 and the Sevenless receptor tyrosine kinase, the adaptor fulfills a critical role in stabilizing and activating a subset of RTKs.

CHIP

In recent years, a few proteins that affect the activity of Hsp70 and Hsp90 have been identified. One of these is the protein CHIP, which binds to Hsp70 and inhibits the client refolding activity of the chaperone (Ballinger et al. 1999). Chip has two domains, a TPR domain and a U-box domain, which implicate it as a candidate for involvement in ubiquitination of proteins through a chaperone-mediated pathway. The U-box domain is homologous to UFD2, a yeast protein that has recently been found to harness E4 activity. This activity is responsible for the generation of multi-ubiquitin chains on substrate proteins (Koegl et al. 1999), leading to their subsequent recognition by the proteasome. In yeast, E4 activity is linked to cell survival under stress conditions and serve a role in mediating degradation of aberrant proteins. In the absence of UFD2, ubiquitination of a substrate is initiated, but only a few ubiquitin molecules are ligated to the substrate protein. This pattern of oligo-ubiquitination is insufficient to recruit the proteasome and cause degradation of ubiquitinated substrates. The other recognizable domain of CHIP, namely the tetratricopeptide repeat (TPR), through which the protein interacts with Hsp70 and Hsp90, is found in several proteins involved in chaperone complexes (Lamb et al. 1995). Consistent with its structural landmarks, CHIP has recently been implicated

in regulation of triage decisions in the context of steroid hormone receptors: through binding to Hsp90 and eliciting release of p23, CHIP abolishes the steroid-binding activity and transactivation potential of the glucocorticoid receptor. Concomitantly, the adaptor induces ubiquitination and degradation of the receptor by the proteasome (Connell et al. 2001). In a similar way, CHIP has been implicated in the ubiquitination and subsequent degradation of the cystic-fibrosis transmembrane-conductance regulator (CFTR) from the endoplasmic reticulum, dependent on Hsp70 and the U-box domain of CHIP (Meacham et al. 2001). Future analyses will address the identity of ubiquitin protein ligases, such as CHIP, that mediate poly-ubiquitination of RTKs through the chaperone pathway.

Concluding Remarks

Many mechanistic aspects of chaperone-mediated ubiquitination and degradation of RTKs remain unknown. The identity of the relevant ubiquitin conjugation enzymes and the respective ubiquitin ligase molecules is one of the major open questions. Not less interesting is the mechanism that so effectively couple chaperones to the ubiquitination machinery. In yeast, ubiquitin's lysine residues 29 and 63 have been implicated in stress resistance (Arnason and Ellison 1994), as well as in endocytosis of a plasma membrane protein (Galan and Haguenauer-Tsapis 1997), which raises the possibility that novel types of ubiquitin branching are associated with the chaperone-mediated pathway in mammals. The Cbl-mediated pathway presents similar open questions. However, the least understood mechanism is the one responsible for constitutive internalization of membrane receptors for either cargo molecules or growth factors. Fragmentary observations and the presence of a ubiquitin interacting motif (UIM) in several molecules involved in constitutive internalization (e.g., Hrs-1, Eps-15 and Epsin) (Hoffman and Falquetb 2001), imply that ubiquitination plays a pivotal role in this pathway, as it does in the other two routes of receptor internalization. Table 1 compares several features of the chaperone-mediated and ligand-induced pathways. It is already clear that the two routes fulfil different physiological requirements and they utilize independent molecular machineries. However, ubiquitin is definitely involved in both pathways,

probably reflecting the existence of a shared core mechanism of vesicular sorting.

Table 1. Comparison of the ligand-induced and the chaperone-mediated pathways of receptor degradation

	Ligand-induced	Chaperone-mediated
Induced by:	Ligand	Stress (and drugs)
Kinase domain	Regulated by kinase function	Regulated by kinase structure
Kinetics	Fast (minutes)	Slow (hours)
ErbB-preference	ErbB-1>>2>3=4	ErbB-2>>1>3=4
Ubiquitin ligase system	c-Cbl	Unknown (CHIP?)
Cellular localization	Plasma membrane and endosomes	Membrane and endoplasmic reticulum
Cellular function	Signal termination	Destruction of misfolded proteins
Relevance of dimerization partner	Highly relevant	Unknown
Degradation	Mostly lysosomal	Mostly proteasomal

Beside their understandable physiological implications, mechanisms that regulate endocytosis and degradation of RTKs have important clinical significance. In many cases membrane bound, as well as cytoplasmic tyrosine kinases impact cell proliferation only when localized to the plasma membrane. Therefore, processes that remove oncogenic tyrosine kinases from the cell surface, and direct them to intracellular destruction, are highly relevant to cancer therapy. Dealing with RTKs of the ErbB family, the *neu* oncogene encodes a constitutively active mutant of the normal Neu/ErbB-2 receptor. By regulating endocytosis and degradation of the oncogenic receptor, c-Cbl acts as a suppressor of *neu* (Levkowitz et al. 2000). Moreover, certain antibodies to ErbB-2 can inhibit the tumorigenic growth of Neu/ErbB-2-overexpressing cancer cells, probably because they

translocate the oncoprotein to an endosomal compartment (Hurwitz et al. 1995) and accelerate its destruction in the lysosome (Maier et al. 1991; Kasprzyk et al. 1992). A similar antibody is currently used to treat patients with metastasizing breast tumors (Baselga et al. 1999). The mechanism underlying immunotherapy seems to involve a c-Cbl-mediated pathway of ErbB-2 ubiquitination and degradation (Klapper et al. 2000b). Another exemplification of the therapeutic implication of RTK ubiquitination and degradation is provided by the ability of Hsp90 antagonists to block the tumorigenic growth of a wide variety of human cancer cells (Schulte and Neckers 1998; Soga et al. 1999). Because Hsp90 controls many clients, the specificity of its antagonists must be limited in order to allow safe clinical use. Potentially, chemical modifications of the inhibitors, such as constructing dimers of geldanamycin, may increase specificity as has been demonstrated for ErbB-2 (Zheng et al. 2000a). These and additional strategies to harness RTK ubiquitination for the benefit of cancer patients will strive on detailed understanding of the multiple mechanisms that control protein stability and propensity to undergo activation.

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Regulation of p27 Degradation

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Cell Cycle Regulation by Cyclin-Dependent Kinases

Progression through the cell cycle depends on the sequential activation of a group of serine/threonine kinases called cyclin-dependent kinases (cdks) (Morgan, 1995; Sherr and Roberts, 1995). Cdk activity is regulated by the integration of positive and negative extracellular signals, which initiate proliferation, cell cycle arrest or differentiation (Sherr, 1996). As the name suggests, cdk function is contingent on its association with an activating cyclin subunit (Draetta, 1994). In mammalian cells, progression through the early G1 phase of the cell cycle is controlled by the activity of cdk4 and cdk6, which associate with D-type cyclins. A salient feature of these cyclin/cdk complexes is their ability to phosphorylate and, so doing, inactivate the retinoblastoma protein (pRb), a step necessary for entry into S phase. Late G1 phase and entry into S phase requires the activity of cdk2, which binds both cyclin E and cyclin A, and further phosphorylates pRb (Figure 1). Once cells have passed the restriction point in late G1 phase, progression through the remainder of the cell cycle will proceed in the absence of mitogenic stimuli (Sherr, 1994).

Mitogenic and anti-mitogenic signals control the activation of cdks, in part, by regulating the cellular abundance of cyclins, acting at the levels of transcription, translation and protein degradation (Draetta, 1994; Sherr and Roberts, 1995). Cdk activity can also be regulated by post-translational

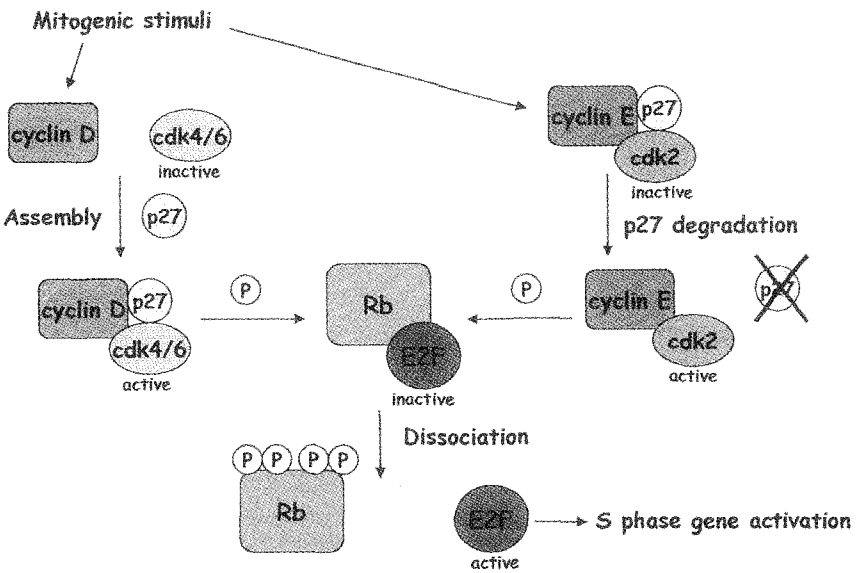

Figure 1. Regulation of S phase entry by p27. p27 facilitates assembly of cyclin D with cdk4 and cdk6 to form active kinases which phosphorylate Rb. Removal of p27 from cyclin E/cdk2 complexes by ubiquitin-dependent proteolysis activates this kinase for further phosphorylation of Rb. Phosphorylated Rb is dissociated from E2F which induces transcription of genes necessary for S phase entry.

modification of the kinase subunit, including phosphorylation by the cdk-activating kinase (CAK), phosphorylation by inactivating tyrosine kinases (Wee1 and Mik1) and dephosphorylation by activating cdk phosphatases (Cdc25a, Cdc25b, Cdc25c) (Morgan, 1995). In addition to positive regulation by binding to cyclins, cdks can be negatively regulated by their association with cyclin-dependent kinase inhibitors (ckis). Two families of ckis, which regulate G1 phase and entry into S phase, have been defined based on sequence similarity and their functional interactions with cdks. The Ink4 family consists of p15, p16, p18, and p20 which block cdk4 and cdk6 (Chan et al., 1995; Guan et al., 1994; Hirai et al., 1995). In contrast, the Kip/Cip family of ckis, which is comprised of p21, p27 and p57, bind to and inhibit the activity of cyclin E/cdk2 and cyclin A/cdk2 complexes (Lee et

al., 1995; Matsuoka et al., 1995; Polyak et al., 1994; Xiong et al., 1993). Interestingly, despite their designation as inhibitors of cdks, p21 and p27 positively regulate cdk4 and cdk6 activity by aiding in the assembly of these proteins with D-type cyclins (Figure 1) (Cheng et al., 1998). The focus of this review will be the regulation of p27 and its deregulation in human cancers.

Features and Functions of p27

p27 was initially identified as an inhibitor of cyclin E/cdk2 and cyclin A/cdk2 in cells arrested in G1 by lovastatin, TGF-β, and contact inhibition (Hengst et al., 1994; Koff et al., 1993; Polyak et al., 1994; Slingerland et al., 1994). It was further determined that levels of p27 protein are elevated in quiescent cells compared to proliferating cells and that levels of p27 protein fluctuate during the cells cycle, with maximal levels occurring during G1 phase (Hengst et al., 1994). The basis for p27-mediated inhibition of cdk2 activity has been elucidated, in part, from the crystal structure of p27 complexed to cyclin A/cdk2 (Russo et al., 1996). Cdk2 is composed of two domains, a small amino-terminal lobe and a larger carboxy-terminal lobe, with the active site of the kinase, where substrate binding and catalysis occurs, contained between these two lobes. Cdk2 is inactive on its own due to a flexible loop (called the T-loop because it contains the Thr-160 CAK phosphorylation site) which blocks substrate binding. Cyclin A contacts both the carboxy-terminal lobe and the T-loop which causes the T-loop to move from its inhibitory position and orients ATP for the kinase reaction (De Bondt et al., 1993). Using a 69 amino acid portion of p27 that is sufficient to inhibit cyclin A/cdk2 activity, Russo et al. characterized the ternary structure of cyclin A/cdk2/p27. The amino-terminus of the p27 peptide binds cyclin A while the carboxy-terminus of the peptide inserts itself within the amino-terminal lobe of cdk2 to disrupt its architecture and block ATP binding within the active site (Russo et al., 1996).

Studies of p27-deficient mice have provided further insight into the function of this cki. In 1996, several groups generated p27 knockout mice and examined the effects on body size, organ development, tumorigenesis, and sterility (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al.,

1996). All three groups found that $p27^{-/-}$ mice are larger than littermate control animals but still exhibit normal body proportions due to concomitant increases in organ size. This effect is both age-dependent, with the difference between wild-type and $p27^{-/-}$ mice becoming more notable with age, and copy number dependent, with $p27^{+/-}$ mice being of intermediate size (Fero et al., 1996). The increased size of p27-deficient mice is consistent with the known role for p27 in cell cycle arrest in response to anti-mitogenic stimuli (Hengst et al., 1994; Koff et al., 1993; Polyak et al., 1994; Slingerland et al., 1994). These mice also display thymic and pituitary hyperplasia and enlarged testes and ovaries, concordant with the high levels of p27 observed in these tissues in normal mice (Nakayama et al., 1996). Similar to mice with mutations in the Rb gene, $p27^{-/-}$ mice frequently develop pituitary tumors, providing further evidence that Rb and p27 function in the same pathway to control G1 arrest (Fero et al., 1996). Loss of p27 results in female infertility, which is attributed to the inability of ovarian follicles to form corpora lutea and abnormal uterine development (Kiyokawa et al., 1996). Despite the link between p27 loss and tumor formation in knockout mice, homozygous inactivating mutations in *CDKN1B*, the gene encoding p27, are seldom found in human tumors. As a result, p27 does not conform to the classical definition of a tumor suppressor protein in which a germline mutation and a somatic mutation occur to inactivate the gene. However, Fero et al. have shown that p27 heterozygous mice are predisposed to tumors when confronted with DNA-damaging agents including γ-irradiation or chemical carcinogens (Fero et al., 1998). This work helped to classify p27 as a haplo-insufficient tumor suppressor because mutation in a single allele renders cells susceptible to tumorigenesis.

Regulation of p27 Protein Levels by the Ubiquitin-Proteasome Pathway

Despite the fluctuations in p27 protein levels during the cell cycle, the amount of p27 mRNA remains unchanged. Instead, p27 protein levels are regulated through translational controls (Hengst and Reed, 1996; Millard et

al., 1997) and degradation by the ubiquitin-proteasome pathway (Pagano et al., 1995). This proteolytic system has been shown to regulate a variety of cellular processes including cell cycle and growth, signal transduction, and metabolic pathways (reviewed in Hershko and Ciechanover, 1998). Proteins that have been conjugated with a chain of ubiquitin molecules are recognized and subsequently degraded by the multicatalyic enzyme, the 26S proteasome. Attachment of a ubiquitin chain to a substrate requires the concerted actions of three enzymes. A ubiquitin-activating enzyme (E1) forms a high-energy thioester bond with ubiquitin in an ATP-dependent reaction. Ubiquitin is subsequently transferred to one of many ubiquitin-conjugating enzymes (Ubcs or E2s). Finally, the ubiquitin moiety is covalently attached to the target protein with the help of a ubiquitin ligase (E3). E3s can function by transferring the ubiquitin from the E2 to the substrate, as in the case of the Hect (for *h*omology to *E*6-AP *C*-*t*erminus) family of ubiquitin ligases. Hect proteins contain a 350 amino acid conserved domain with a cysteine residue which forms a thiolester intermediate with ubiquitin prior to transfer to the substrate (Jackson et al., 2000; Kumar et al., 1997). Alternatively, E3s can bring the E2 in proximity of the target for direct transfer of ubiquitin from the E2 to the substrate, as in the case of RING finger-dependent ligases (Rdls). RING fingers contain Cys and His residues responsible for coordinating zinc ions. Although it is not yet clear how proteins containing RING fingers promote ubiquitination, but many RING finger proteins bind E2s and have been demonstrated to be critical for efficient ubiquitination of specific substrates (Jackson et al., 2000).

Several years ago, p27 was demonstrated to be a substrate of the ubiquitin-proteasome pathway both in vivo and in vitro (Pagano et al., 1995). Treatment of MG-63 human osteosarcoma cells with inhibitors of the chymotryptic activity of the proteasome results in an increase in p27 levels. Furthermore, higher molecular weight species of p27 can be detected in cells treated with proteasome inhibitors with a ubiquitin-specific antibody. In vitro, purified recombinant p27 can be ubiquitinated in the presence of rabbit reticulocyte extract. Importantly, in vitro systems for p27 ubiquitination and degradation have implicated Ubc3 (also called Cdc34) as the specific E2 enzyme for p27 turnover. Another significant finding is that proliferating cells have a much greater capacity for p27 ubiquitination than

quiescent cells, which accounts for the elevated levels of p27 observed in G0 cells.

Later evidence suggested that phosphorylation of p27 is a prerequisite for its degradation by the ubiquitin-proteasome pathway. p27 phosphorylation was shown to be cell cycle dependent, with a peak in late G1 phase. A cdk phosphorylation consensus site (Thr-Pro-Lys-Lys) was identified in p27 (Vlach et al., 1997), and threonine-187 was confirmed as a phosphorylation site by cyclin E/cdk2 complexes in vitro (Sheaff et al., 1997). Ectopic expression of p27 mutated in its cdk phosphorylation site (Thr-187-Ala) is a stable protein compared to wild-type (Nguyen et al., 1999). Further in vitro studies demonstrated that either depletion of cdks or the use of the unphosphorylable p27 mutant (Thr-187-Ala) ablates p27 ubiquitination, thus establishing the importance of cdk-dependent phosphorylation on threonine-187 for p27 turnover (Montagnoli et al., 1999). In addition, the cell cycle stage specificity of p27 ubiquitination and degradation has been examined. Recombinant p27 can be ubiquitinated in vitro by extracts from proliferating cells, but not from G1-enriched cells (Montagnoli et al., 1999; Nguyen et al., 1999). In vivo studies have demonstrated that p27 is phosphorylated on threonine 187 and subsequently ubiquitinated in proliferating cells but not in G1 cells (Muller et al., 1997; Sheaff et al., 1997). These studies explain the high levels of p27 observed in G1 cells compared to other phases of the cell cycle.

Regulation of the G1/S Transition by SCF Complexes

Once it was established that p27 was regulated by ubiquitin-dependent proteolysis, research focused on the identification of the ubiquitin ligase responsible for p27 ubiquitination. Three lines of evidence, 1) p27 is stable in G0 and early G1 phase, 2) p27 phosphorylation is necessary for ubiquitination and 3) Ubc3/Cdc34 promotes the ubiquitination of p27, suggested that ubiquitination of p27 is accomplished by a specific set of E3 enzymes called SCF complexes (reviewed in DeSalle, 2001). These multiprotein ligases are so called, because they are composed of Skp1, Cul1, an F-box protein (Fbp) and Roc1/Rbx1. They belong to the Rdl family of E3s because Roc1 contains a RING-H2 finger domain and promotes the

association of Cul1 with the E2 (Kamura et al., 1999; Ohta et al., 1999). While ubiquitin-proteasome mediated degradation is crucial for the regulation of cell cycle progression, SCF complexes are of particular importance for entry into S phase (DeSalle, 2001). Furthermore, phosphorylation of the substrates is required for recognition by SCF enzymes to target them for ubiquitin-dependent proteolysis (Bai et al., 1996). Finally, SCF complexes often function with the E2, Ubc3/Cdc34, in the ubiquitination reaction (Lisztwan et al., 1998).

It is well documented in yeast that SCF complexes are key in controlling the cellular abundance of cell cycle regulatory proteins. In *S. cerevisiae*, entry into S phase requires the activity of Cdk1-Clb5 and Cdk1-Clb6 kinases. The Cki Sic1 is a negative regulator of these kinases and therefore must be degraded before DNA replication can occur (Mendenhall, 1993; Schwob et al., 1994). Genetic and biochemical studies revealed that proteolysis of Sic1 is controlled by the ubiquitin conjugating enzyme Ubc3/Cdc34 and by an SCF ubiquitin ligase formed by four subunits: CulA (also called Cdc53), Skp1, Hrt1 (the homolog of Roc1/Rbx1) and the F-box protein Cdc4 (Schneider et al., 1996; Tyers, 1996). Three SCFs that are well characterized so far in *S. cerevisiae* are: SCF^{Cdc4} (which recruits as substrates the Ckis Sic1 and Far1, the replication factor Cdc6, and the transcriptional activator Gcn4), SCF^{Grr1} (which recruits the G1 cyclins Cln1 and Cln2), and SCF^{Met30} (which recruits the Cdc28 inhibitory kinase Swe1 and appears to be involved in the degradation of transcription factors regulating methionine biosynthesis) (reviewed in Patton et al., 1998). It is the Fbp component of the SCF complex that is responsible for substrate recognition. Fbps are defined by a 40 amino acid motif called the F-box (because it was first identified in cyclin F) (Bai et al., 1996) and characterized by additional protein-protein interaction domains such as WD-40 repeats or leucine-rich repeats. Skp1 binds the Fbp through its F-box to link the Fbp to Cul1, Roc1/Rbx1 and the ubiquitination machinery. In turn, the Fbps recruit phosphorylated substrates through their divergent protein-protein interaction domains. This system allows for specific recognition of a variety of substrates by SCF complexes through different F-box proteins (reviewed in Pagano, 1997; Patton et al., 1998).

SCF^{Skp2}

In humans, 6 genes are known that are homologous to yeast CulA: Cul1, Cul2, Cul3, Cul4A, Cul4B and Cul5. While all of these gene products are capable of binding to oc1/Rbx1, only Cul1 can bind Skp1 to form an SCF complex (Lyapina et al., 1998; Michel and Xiong, 1998). A number of mammalian Fbps have been identified through yeast two-hybrid screenings and database searches (Cenciarelli et al., 1999; Winston et al., 1999).

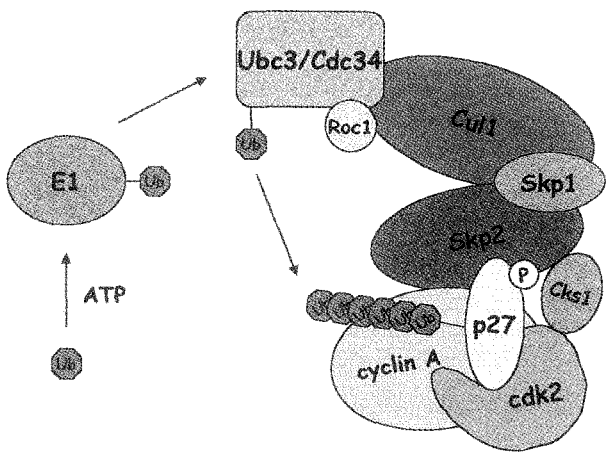

Figure 2. Schematic representation of SCF^{Skp2}. Phosphorylated p27 is recognized by Skp2 and ubiquitinated by the concerted actions of E1, Ubc3/Cdc34 and SCF^{Skp2}. Cks1 is an accessory protein that enhances binding of phosphorylated p27 to Skp2 and is required for efficient ubiquitination of p27 by SCF^{Skp2} (see text for a more detailed description).

However, the majority of these Fbps have not been characterized and their substrates remain unknown. Human Skp2 is an Fbp that was originally identified, along with Skp1, as a binding partner of cyclin A/cdk2 (Zhang et al., 1995). Thus, the proteins were named S-phase kinase associated proteins. It was later shown that similarly to yeast SCF complexes, Skp1 and Skp2 associate with Cul1 to form an evolutionarily conserved SCF complex

(Lisztwan et al., 1998). Skp2 is a v cell cycle-regulated protein; Skp2 accumulates at the G1/S transition and peaks during S phase, while Cul1, Skp1, Roc1, and Ubc3/Cdc34 levels do not change (Carrano et al., 1999; Marti et al., 1999).

In 1999, several groups identified Skp2 as the Fbp responsible for recognition of phosphorylated p27 for ubiquitination (Carrano et al., 1999; Sutterluty et al., 1999; Tsvetkov et al., 1999). Skp2 is able to interact with phosphorylated p27 both in vitro and in vivo. Moreover, the addition of recombinant Skp1/Skp2 complexes to G1 cell extract can restore in vitro ubiquitination of p27 to levels similar to those obtained with proliferating cell extracts. Overexpression in mammalian cells of a Skp2 mutant with the F-box deleted that can no longer recruit the ubiquitination machinery results in a stabilization of overexpressed p27, while treatment of cells with antisense Skp2 oligonucleotides to target skp2 mRNA stabilizes endogenous p27. Finally, a Skp2 knockout mice were generated which are viable; however, $Skp2^{-/-}$ cells have increased levels of p27 and free cyclin E (not bound to cdk2) (Nakayama et al., 2000). Taken together, these results implicate SCF^{Skp2} as the E3 ligase responsible for p27 ubiquitination and subsequent degradation (Figure 2). In addition to p27, several other Skp2 substrates have been suggested including p21, cyclin D1, cyclin A, and E2F-1 (Nakayama et al., 2000; Yam et al., 1999; Yu et al., 1998). However, only p27 and free cyclin E but not other suggested substrates accumulate in Skp2 deficient cells (Nakayama et al., 2000). This indicates either that p27 and cyclin E are the only substrates of Skp2 or that additional substrates are degraded by more than one pathway. Importantly, all cellular and histopathological abnormalities observed in Skp2 deficient mice are abolished in Skp2/p27 double knock-out mice (Nakayama K. and Nakayama K. I., personal communication), indicating that p27 is a primary substrate of Skp2.

More recent work has concentrated on the regulation of Skp2 levels during the cell cycle (Carrano and Pagano, 2001; Wirbelauer et al., 2000). Wirbelauer et al. examined Skp2 mRNA and protein levels during re-entry of quiescent cells into the cell cycle. They found that while Skp2 protein is lacking in G0/G1 cell and accumulates at the initiation of S phase, Skp2 mRNA levels exhibited only minor changes as cells progressed from G1 to S phase. To understand how Skp2 is regulated during G0/G1, human diploid

fibroblasts were arrested by serum deprivation or induced to re-enter the cell cycle by the addition of serum in the presence or absence of the proteasome inhibitors LLnL or MG132. Addition of proteasome inhibitors to serum-starved cells induced an increase in Skp2 levels, suggesting that in the absence of mitogen, Skp2 is regulated by proteasome-dependent degradation. This explains the absence of Skp2 in quiescent cells.

In addition to regulation by growth factor stimulation, Skp2 levels are controlled by adhesion to the extracellular matrix. Carrano et al. found that Skp2 is expressed in adherent cells stimulated to enter the cell cycle but is absent when cells in suspension are reactivated with serum (Carrano and Pagano, 2001). This result correlates with p27 levels, which decrease as adherent cells enter S phase, but are stable in suspension cells. Retroviral expression of Skp2 in cells plated in suspension induces cells to degrade p27 and enter S phase. These data suggest an important role for extracellular matrix signaling in the regulation of Skp2 levels. Interestingly, cell adhesion regulates Skp2 levels at the level of transcription, by inducing Skp2 mRNA accumulation, without affecting proteasome-mediated degradation of Skp2 protein. Thus, growth factors and the extracellular matrix cooperate to upregulate Skp2 by different mechanisms.

Cks1

Despite the identification of SCF^{Skp2} as the ligase that ubiquitinates p27 in vivo, ubiquitination of p27 in vitro using purified components of the specific ubiquitination machinery could not be reconstituted until recently. In contrast, ubiquitination of the yeast SCF substrate, Sic1, as well as ubiquitination of IκBα by human $SCF^{\beta TrCP}$ can be achieved in vitro (Feldman et al., 1997; Skowyra et al., 1997). Several groups, using very different approaches, identified cdc kinase subunit 1(Cks1) as an essential component of the p27 ubiquitination machinery (Ganoth et al., 2001; Spruck et al., 2001). Cks proteins are small proteins (9-18 kD) which have important roles in progression through the G1/S and G2/M phases of the cell cycle and in exit from mitosis. Suc1, a Cks protein in fission yeast, was the first to be identified as a suppressor mutant of the cdk, cdc2 (Hayles et al., 1986). Later, homologs were identified in budding yeast, Cks1 (Hadwiger et

al., 1989), and in humans, Cks1 and Cks2 (Richardson et al., 1990). Studies in fission and budding yeast and in *Xenopus* extracts suggested that Cks proteins had a primary role in G2/M phase because ablation of Cks1 function causes defects in entry into and exit from mitosis (Patra and Dunphy, 1996; Tang and Reed, 1993). Cks1 functions in this capacity, at least in part, by binding to cyclin B/cdk1 and assisting in the multiple phosphorylation of mitotic substrates (i.e. Cdc25, Wee1and Cdc27). Cks proteins were determined to be general interactors of cdks without directly promoting or inhibiting the catalytic activity of cdks. Instead, they appear to promote multiple phosphorylations on the same substrate. Crystal structures of the fission yeast and human Cks proteins have been solved (Bourne et al., 1996; Pines, 1996). Predicted models of Cks complexed to cyclin/cdks indicate that Cks might function by extending the site of interaction for cdk substrates and perhaps by inhibiting substrate release after phosphorylation. This mechanism underlies the mitotic function of Cks proteins.

It was known that the Cks proteins have an additional role in the G1 to S transition because yeast lacking Cks1 arrest in G1 (Tang and Reed, 1993). However, the mechanism by which Cks proteins function during G1/S was unclear. The only established effect of Cks1 during G1/S came from Cks1-deficient yeast which displayed lower levels of kinase activity toward the ckis Sic1 and Far1 (Reynard et al., 2000). More recent work has confirmed a role for Cks proteins in the G1/S transition. As a collaborative effort, Ganoth et al. used a biochemical approach to assay fractions of HeLa extract for their ability to promote p27 ubiquitination in the presence of purified SCF^{Skp2}, cyclin E/cdk2, Ubc3 and E1. The factor that was able to stimulate ubiquitination was purified to homogeneity and identified as Cks1. Including Cks1 in an in vitro reaction with E1, Ubc3, Cul1, Skp1, Skp2, Roc1, ubiquitin and cyclin E/cdk2 permits reconstitution of p27 ubiquitination with purified components. It was determined that the function of Cks1 in the ubiquitination reaction is to facilitate binding of phosphorylated p27 to Skp2. At the same time, Spruck et al. generated Cks1 knockout mice and found that these mice were smaller than wild-type littermates. Analysis of $Cks1^{-/-}$ MEFs revealed that these cells had elevated levels of p27. Extracts from $Cks1^{-/-}$ MEFs are defective in their ability to ubiquitinate p27. Furthermore complexes of cdk2/cyclin A/p27 or cdk2/cyclin E/p27 do not associate with Skp2 in $Cks1^{-/-}$ cells while

tetrameric complexes can be detected in wild-type cells. These results show that Cks1 is critical for p27 ubiquitination and the G1/S transition and provide the first instance of reconstitution of ubiquitination of an SCF target requiring an accessory protein. Importantly, Cks1 binds Skp2 and cdk2, Skp2 interacts with cyclin A/E, p27 and Cks1, while p27 has contacts with cyclin A/E, cdk2, and Skp2 (Figure 2). In this complex, Cks1 may function by altering the conformation of Skp2 to promote binding to phosphorylated p27. Alternatively, Cks1 may act as an adaptor protein by binding Skp2 and the phosphate group of p27 phosphorylated on Thr187 simultaneously. Multiple sites of contacts within this complex likely facilitate the ubiquitination of phosphorylated p27 by SCF^{Skp2} and explain why p27 in a trimeric complex is a better substrate for ubiquitination than free p27 (Montagnoli et al., 1999).

Skp2 and Oncogenesis

Based on its characterized activity and the tumor phenotype observed in knockout mice, p27 is a tumor suppressor protein. However, mutations in the gene encoding p27 are rarely observed in human malignancies. Instead, loss or reduction of p27 protein has been observed in many human cancers, and this deficiency of p27 reflects increased proteasome-mediated degradation (Chiarle et al., 2000; Esposito et al., 1997; Loda et al., 1997; Piva et al., 1999). Recent evidence suggests that, due to its role in the degradation of the tumor suppressor p27, Skp2 has oncogenic potential. Several reports have evaluated the correlation between Skp2 expression and grade of malignancy in human tumors (Gstaiger et al., 2001; Hershko et al., 2001; Latres et al., 2001). Latres et al. examined Skp2 expression by immunohistochemistry in low- and high-grade human lymphoid malignancies and found that a direct correlation exists between Skp2 expression and high-grade malignancy. These criteria inversely correlate with p27 levels. Furthermore, high Skp2 levels correspond to expression of cell proliferation markers. Similarly, Gstaiger et al. evaluated Skp2 expression during progression from oral epithelial dysplasia to invasive carcinoma. They found that Skp2 is overexpressed in epithelial dysplasias and carcinomas and that Skp2 levels increase with higher grade epithelial

dysplasias. Finally, an inverse correlation between Skp2 and p27 expression can be observed in oral tissues and Skp2 expression correlates with poor prognosis of oral cancer (Y. Kudo, personal communication). Together, these results suggest that Skp2 overexpression may contribute to p27 deregulation in lymphomas and oral epithelial dysplasias and carcinomas. Moreover, Skp2 may serve as a useful prognostic marker for human cancers.

These groups further examined the oncogenic potential of Skp2 in vivo. Transgenic mice were generated with Skp2 targeted to T cell lineage. Skp2 transgenic mice were healthy and did not develop tumors (Latres et al., 2001). To determine if Skp2 can cooperate in tumorigenesis, these mice were crossed with transgenic mice carrying an activated N-Ras gene. Seventy-five percent of double transgenic mice developed T cell lymphomas compared to twenty-five percent of mice carrying only the N-Ras transgene,

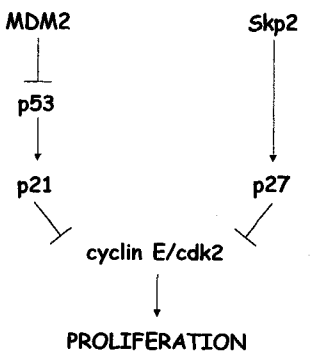

Figure 3. Oncogenic potential of ubiquitin ligases. MDM2 and Skp2 regulate levels of the tumor suppressors p53 and p27, respectively. Deregulated degradation of p53 and p27 results in cyclin E/cdk2 activation and cell proliferation.

and these mice died at a much faster rate than mice carrying the N-Ras transgene alone. Analysis of these tumors revealed decreased levels of p27 as compared to normal extracts. Gstaiger et al. analyzed Skp2 for its ability to transform primary rat embryo fibroblasts (REFs). Cotransfection of Skp2 and H-Ras in REFs yield transformed foci. Transformants display significantly lower p27 levels compared to controls. Furthermore, Skp2/H-Ras transformed cells are capable of inducing tumor formation when

injected into nude mice. A third study examined the effect of overexpression of both Skp2 and cyclin E on hepatocyte proliferation. Using recombinant adenoviral vectors to ectopically express Skp2 and cyclin E in cultured hepatocytes, Nelsen et al. found a synergistic effect on cell proliferation in the absence of mitogenic stimuli. Furthermore, cotransfection of Skp2 and cyclin E triggered liver hyperplasia. In sum, these results indicate that Skp2 has an oncogenic capacity and may cooperate with Ras to promote cell transformation and tumor formation in human cancers.

Conclusions

It has been evident for several years that p27 is a critical regulator of the cell cycle and that loss of p27 protein or function is an important step in the development of many human cancers. Only recently, has the regulation of p27 levels by the ubiquitin-proteasome pathway been elucidated. It is clear that multiple levels of regulation contribute to the timed degradation of p27, including phosphorylation of p27 on Thr-187 by cyclin E/cdk2 and cyclin A/cdk2 and relative levels of SCF^{Skp2} ubiquitin ligase components. For instance, cyclins A and E and Skp2 are induced by growth factors, while cell adhesion result in increased cyclin A and Skp2 levels (Carrano and Pagano, 2001; Sherr and Roberts, 1995; Wirbelauer et al., 2000). Cks1 transcription is upregulated by growth factors but down-regulated by TGF-β stimulation (Richardson et al., 1990; Simon et al., 1995). Furthermore, Cul1 levels are decreased by cell-cell contact inhibition (O'Hagan et al., 2000). Despite decreases in Skp2 levels observed in conditions including lack of cell adhesion (Carrano and Pagano, 2001), increased PTEN levels (Mamillapalli et al., 2001), or increased Rb levels (L.Zhu, personal communication), p27 remains phosphorylated on Thr-187. This suggests that numerous pathways must be coordinated for the precise destruction of p27 during S phase and that the redundancy of p27 regulation is necessary to properly respond to different stimuli including growth factors, cytokines, and adhesion conditions. The gene products that specifically regulate p27 protein abundance are also of significance in cellular transformation. Indeed, Skp2 has the potential to act as an oncoprotein by stimulating cell proliferation through p27 degradation. Similarly, Cks1 may have oncogenic potential

based on its ability to facilitate the ubiquitination of p27. It is clear that ubiquitin ligases can also be oncoproteins. For instance, MDM2 was initially characterized as an oncogene (Keleti et al., 1996; Khatib et al., 1993) and later found to negatively regulate the tumor suppressor p53 by acting as a ubiquitin ligase and targeting p53 for ubiquitin-dependent proteolysis (Figure 3) (Honda et al., 1997). Future research will doubtlessly focus on the pathways regulating Skp2 and Cks1 abundance. Due to their specificity of function, Skp2 and Cks1 may serve as important prognostic markers for various malignancies and these proteins may be useful therapeutic targets for human cancers.

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Ubiquitin System-Dependent Regulation of Growth Hormone Receptor Signal Transduction and Effects of Oxidative Stress

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The Cytokine/Hematopoietin Receptor Superfamily

The GHR was initially cloned from rabbit and human cDNA libraries and subsequently from other species, presenting amino acid sequence homologies of ~70% between the different species (Leung et al., 1987). The rabbit GHR contains a total of 620 amino acid residues, of which 246 are part of the extracellular domain, 24 of the transmembrane domain, and 350 of the intracellular domain. In addition to the membrane-bound form of GHR, a soluble circulating form of the receptor, named growth hormone binding protein (GHBP), has been characterized. In general, the GHBP is generated by proteolytic cleavage at the cell surface (shedding) of the membrane-bound form of the receptor by a recently identified metalloprotease called tumor necrosis factor (TNF)-α-converting enzyme (TACE or ADAM-17) (Zhang et al., 2000). Diverse biological functions have been attributed to the GHBP. In plasma, complex formation of GHBP-GH creates a circulating GH reservoir, protects GH from degradation and excretion, prolongs its half-life, and may enhance its bioactivity in vivo through these mechanisms. Serum GHBP is an useful tool for measuring GHR abundance in the body (Baumann, 1995).

Growth hormone (GH), also termed somatotropin or somatotrope hormone, is a polypeptide hormone essentially secreted by the pituitary gland that exerts its effects via cell surface specific receptors located at the surface of target issues. GH has long been known to mainly promote postnatal longitudinal body growth and differentiation of muscle, bone, and cartilage cells (Isaksson et al., 1985). Through the interaction with the GHR, GH regulates the lipid, carbohydrate, nitrogen and mineral metabolism within a cell. Many of the actions of GH are mediated by the activation of insulin-like growth factor one (IGF-1). Hypersecretion of the hormone can lead to giantism and acromegaly in adults. Chronic GH treatment increases GH binding in hepatic tissues, upregulates the number of GH binding sites at the cell surface but, at the same time, initiates GHR downregulation (Lesniak and Roth, 1976). Mal-nutrition and fasting lead to a state of GH resistance characterized by increased circulating levels of GH and decreased IGF-1 concentrations. These hormonal changes are accompanied by a decrease in GHRs in hepatic membranes (Straus and Takemoto, 1990).

Signaling Mechanisms of the GHR

Studies on the crystallographic structure of GH-GHR complexes revealed a trimeric complex of two receptors and a single molecule of hormone (De Vos, et al., 1992). Analysis of the complex revealed two binding sites on hGH on opposite sides with slightly different binding affinities (Waters et al., 1994). The ligand binding domain and dimerization interface at the extracellular part of the GHR lie on its cystein pairs and YGEFS motif. The GH binding to the two receptors is considered the first step in the action of GH. This process is initiated with GH binding to one molecule of receptor through its site 1, a functional epitope of 11 amino acids, followed by association of this complex to a second receptor molecule through GH's site 2, a 31 amino acid region that stabilizes and, together with a ~500 Å contact region between the two extracellular domains of GHR, defines the binding of the second GHR (Reviewed in Wells et al., 1993). Receptor dimerization is crucial for signal transduction since a hGH antagonist, G120R, still being able to bind to the first GHR via its site 1 but defective in its site 2 to induce

dimerization, cannot transduce signal, being however internalized as efficiently as the wild type ligand (Fuh et al., 1992; Harding et al., 1996).

GHR Dimerization and JAK2 Activation

Although the GHR and other cytokine receptors do not possess intrinsic tyrosine kinase activity, GH binding to its receptor results in rapid tyrosine phosphorylation of multiple cellular proteins due to the activation of GHR-associated tyrosine kinases of the JAK family. The ability to associate with and activate tyrosine kinases is essential for the ability to propagate most intracellular signals (Fig. 1). The JAK kinases represent a distinct family of soluble tyrosine kinases that have been strongly implicated in the signal transduction of many members of the cytokine family (Reviewed in:Argetsinger and Carter Su, 1996). Among these known members of the JAK family, JAK2 is the main kinase activated by the GH:GHR complex and considered the initiating step in GHR signal transduction (Argetsinger et al., 1993). JAK2 binds to the GHR via box-1, the proline-rich region in the cytoplasmic domain (Colosi et al., 1993). No specific amino acid within box-1 is essential for the association between the receptor and JAK2. Mutation of each individual proline residue in box-1 or the simultaneous mutation of the first two prolines did not impair the kinase association. However, a specific secondary structure of the receptor is required, in which simultaneous mutation of the last two proline residues or of the three hydrophobic residues (isoleucine; leucine and valine into threonine) abolishes the capacity of the receptor to interact with and to activate JAK2 (Dinerstein et al., 1995). The box-1 sequence of the GHR is very similar to proline-rich SH3 domains. However, no SH3 binding domain has been identified in JAK2, and the existence of an SH3-containing adaptor protein could mediate the association. The first 46 residues of the GHR cytosolic tail, containing the box-1, are sufficient to induce some JAK2 activation, but maximal activity of this kinase requires downstream residues in the half-proximal transmembrane part of the GHR cytoplasmic domain. In resting cells, JAK kinases are thought to be associated with the cytoplasmic domains of receptor, but catalytically inactive. Upon ligand-stimulation, the GHR becomes firmly dimerized, initiating enzyme activity in two JAK2

molecules, which trans-phosphorylate each other on one or more tyrosine residues within the kinase domain of the paired JAK2.

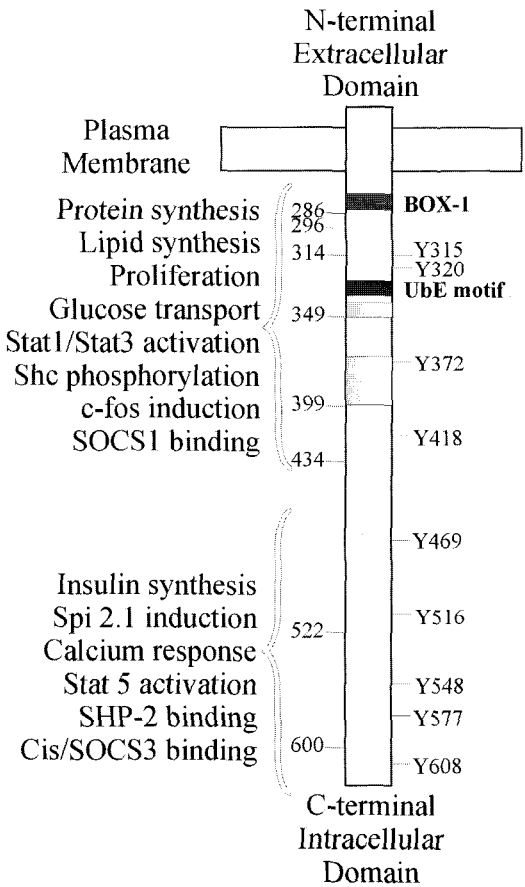

Figure 1. Structure of the GHR cytosolic tail

GHR Signaling Pathways

Upon GH binding to its receptor, activation of JAK2 is rapid and transient, reaching maximal activity at 5-20 min and return to basal levels after 60 min. Once activated, JAK2 phosphorylates the GHR on multiple tyrosine

residues providing docking sites for other signaling molecules (Wang et al., 1996). Some of the effects of GH are mediated directly through JAK2, such as stimulation of cell proliferaton (Colosi, et al., 1993).

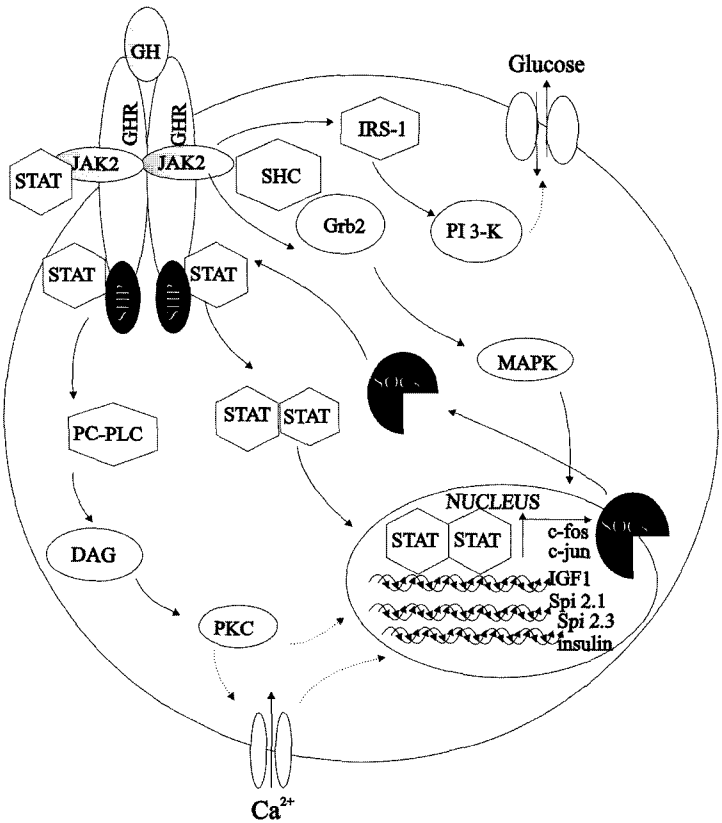

Figure 2. GHR Signal Transduction Pathway. Black images represent negative regulators of GHR signaling.

JAK2 contains 48 tyrosine residues, which all might become phosphorylated upon GH, suggesting that this kinase can interact with multiple signaling molecules (Silvennoinen et al., 1993). Among the direct substrates of JAK2 are several docking proteins involved in the Ras/MAP kinase pathway, the STAT proteins (signal transducers and activators of transcription), as well as the insulin receptor substrate proteins IRS-1 and

IRS-2, which initiate the PI-3 kinase pathway (Reviewed in:Carter Su and Smit, 1998). So far, only GH-dependent calcium transport appears to involve a pathway that is independent of JAK2 activation (Billestrup et al., 1995). Other GH effects like GH-dependent expression of the insulin gene or the serine protease inhibitor (Spi) 2.1 gene require additional sequences in the GHR cytoplasmic domain (Goujon et al., 1994; Moldrup et al., 1991). In a more distal C-terminal part of the GHR cytoplasmic domain, phosphorylation of Y487, Y534, Y566 or Y627 is required for tyrosine phosphorylation of STAT5, while Y333 and/or Y338 GHR appear to be required for GH-stimulated lipid and protein synthesis (Lobie et al., 1995; Xu et al., 1996).

Signal Transducers and Activators of Transcription

STAT proteins are a family of transcription factors that couple ligand binding to cellular receptors with the activation of gene transcription (Darnell et al., 1994). Currently, the sequence of seven genes from mammals that encode STAT family members have been reported (Reviewed in:Finidori, 2000). GH was found to be able to activate STAT1, STAT3 and both isoforms of STAT5, a and b (Smit et al., 1996). Upon JAK2 tyrosine phosphorylation, cytoplasmic STAT proteins are recruited through their SH2 domain in the complex GHR/JAK2 kinase and are subsequently phosphorylated by JAK2 on their conserved C-terminal tyrosine. Activated STATs homo- or heterodimerize with other STATs and/or non STAT proteins, presumably via their SH2 and SH3 domains, translocate to the nucleus, bind DNA and activate transcription of target genes (Ihle, 1996). STAT1 and STAT3 are phosphorylated and bind to the c-sis-inducible element (SIE) of the c-fos promoter/enhancer (Gronowski and Rotwein, 1994). Similarly, GH promotes the binding of STAT5 to the GH-responsive element 2 (GHRE-2) in the promoter region of the Spi 2.1 gene (Goujon, et al., 1994). In CHO cells expressing recombinant GHR, it was shown that GH induced tyrosine phosphorylation of both STAT1 and STAT3 (Strous et al., 1997). The regions of the GHR required for activation of the different STATs has been mapped: STAT1, STAT3 and STAT5 all require the membrane proximal part of the GHR including box-1, and JAK2 activation

GH in order to become phosphorylated and activated (Gronowski and Rotwein, 1994; Sotiropoulos et al., 1995).

MAP Kinases

The main signaling pathways stimulated by GH are shown in Fig 2. Two such proteins that are activated by GH are the MAP kinases ERK1 and ERK2 (Anderson, 1992; Campbell et al., 1992). MAP kinases are a family of the serine/threonine/tyrosine kinases that mediate both cellular growth and differentiation upon stimulation by many growth factors and hormones. One pathway involves SHC tyrosine phosphorylation followed by the sequential recruitment of Grb2, Son-of-sevenless (Sos), Ras, Raf, and MAP kinase kinase (MEK) (VanderKuur et al., 1997; Winston and Hunter, 1995). GH rapidly and transiently stimulates tyrosine phosphorylation of the 46-, 52-, and 66-kDa known isoforms of SHC. It is thought that JAK2 phosphorylates SHC, which in turn interacts with and activates growth factor receptor bound to Grb2. Grb2 activates RAS, RAF, MEK and MAPK via Sos (VanderKuur, et al., 1997). MAPK activation by GH may be mediated via activation of the epidermal growth factor (EGF) receptor (Yamauchi et al., 1997). It has been reported that EGFR was phosphorylated by JAK2 and may act as a docking protein for Grb2. This finding is a clear example of cross-talk between membrane receptors of different families.

Insulin Receptor Substrates-1 and –2 (IRS-1 and IRS-2) and Phosphatidylinositol 3-kinase (PI3-K)

Upon GH stimulation, rapid insulin-like effects like increased amino acid transport, glucose transport and lipogenesis occur (Davidson, 1987). The finding that GH can interact with IRS-1 and induce its phosphorylation was observed in rat adipocytes, in 3T3-F442A fibroblasts and in CHO cells expressing GHR (Souza et al., 1994). IRS-2 was also reported to associate to GHR complexes (Argetsinger et al., 1996). IRS-1 and IRS-2 are large cytosolic proteins (160-180 kDa) that appear to serve as mobile adapter proteins. They are phosphorylated on multiple tyrosine residues in response to insulin stimulation and form high-affinity binding sites for signaling

molecules like the p85 regulatory subunit of phosphatidylinositol kinase, the SH2 domain-containing tyrosine phosphatase two (SHP2), the Src family kinase fyn, Nck, and Grb2 (Argetsinger, et al., 1996). The nature of the GH-stimulated interaction with IRS-1 is still not clearly understood. Studies using truncated and mutated GHRs expressed in CHO cells showed that the regions of the receptor required for tyrosine phosphorylation of IRS-1/IRS-2 were the same as that required for JAK2 activation (Argetsinger, et al., 1996). Tyrosine phosphorylation of IRS-1 and IRS-2 depends on JAK2 activation, and the interaction of the GHR-JAK2 complex with IRS-1 and IRS-2 occurs through activated JAK2 either directly or via an intermediary protein. GH promotes the binding of the 85-kDa regulatory subunit of PI 3-kinase to IRS-1 and IRS-2 (Argetsinger, et al., 1996). PI 3-kinase is implicated in several pathways, including DNA synthesis, glucose uptake, cell cycle regulation via p70 ribosomal subunit kinase (rsk) activation, and inhibition of apoptosis via Akt serine threonine kinase (Cheatham et al., 1994; Songyang et al., 1997). Most cytokine receptors induce the recruitment of PI 3-kinase in the activated cytokine receptor complex, the interaction being direct or indirect. In the case of the GHR, PI 3-kinase could associate with either the receptor, JAK2, or IRS-1 and IRS-2. However, PI 3-kinase is activated but not tyrosine phosphorylated under GH stimulation. The role of PI 3-kinase in GHR signaling could partially explain the insulin-like effects of GH as wortmannin, a PI 3-kinase inhibitor, blocks the ability of GH to stimulate lipid synthesis (Ridderstrale et al., 1995).

PLC, PKC, Ca^{2+} Pathways

GH has been found to activate protein kinase C (PKC) through the phospholipase C (PLC) signal transduction pathway. Activation of PKC by GH is thought to stimulate lipogenesis, induce c-fos expression, increase intracellular Ca^{2+} concentration, and stimulate binding of nuclear protein to C/EBP oligonucleotide. For most of the PKC isoforms, activation and translocation from the cytosol to the cellular membrane is mediated by the second messenger Ca^{2+} and/or 1,2-diacylglycerol (DAG). GH transiently induces activation of DAG, an activator of PKC, in multiple cell types, that

can then bind, recruit, and stimulate PKC (Doglio et al., 1989). Ca^{2+} increase by GH seems to be dependent on L-type calcium channel activation by a mechanism including phospholipid hydrolysis and PKC activation (as mentioned above). Ca^{2+} is important for activation of some GH-induced genes such as the Spi2.1 gene and the effects of GH on metabolism in adipocytes (Billestrup, et al., 1995). Ca^{2+} activation is until now the only pathway activated by GH that does not require Box-1 but only the C-terminal part of the GHR (Billestrup, et al., 1995).

Negative Regulators of GH Signaling

Phosphatases

Pre-incubation with pervanadate, which inhibits phosphatase activities, resulted in a prolonged JAK2 and STAT5 tyrosine phosphorylation (Gebert et al., 1999a), indicating that phosphatases are involved in the inactivation process. Truncation of the C-terminal region of the GHR tail, sustained JAK2 phosphorylation and enhanced STAT3 and IRS-1 activation, suggesting that a phosphatase may interact with the C-terminal cytoplasmic domain of the GHR (Alves dos Santos et al., 2001b; Sotiropoulos et al., 1996). Indeed, the tyrosine phosphatase SHP-1 was found to interact with JAK2 and to dephosphorylate the kinase (Hackett et al., 1997; Ram and Waxman, 1997). Recently, the transmembrane PTPase CD45, a key regulator of antigen receptor signaling in T and B cells, was found to suppress JAK kinases and negatively regulate cytokine receptor signaling (Irie-Sasaki et al., 2001; Kishihara et al., 1993). Targeted disruption of the CD45 gene lead to enhanced cytokine and interferon-receptor-mediated activation of JAKs and STAT proteins. *In vitro*, CD45 directly dephosphorylated and bound JAKs and was shown to negatively regulate interleukin-3-mediated cellular proliferation, erythropoietin-dependent haematopoiesis and antiviral responses *in vitro* and *in vivo*.

Kinase inhibitors such as H7, which inhibits serine threonine kinases, resulted in sustained JAK2/STAT activation, suggesting that besides protein dephosphorylation, protein phosphorylation is also required. Furthermore, both inhibition of protein synthesis with cycloheximide and inhibition of

protein degradation with proteasome inhibitors such as MG132 induced prolongation of the signal (Fernandez et al., 1998; Gebert et al., 1999b). Therefore, several processes are probably involved in GHR signal downregulation that require both activation of phosphatases, activation of kinases, protein degradation and protein biosynthesis (Alves dos Santos, et al., 2001b).

Suppressor of Cytokine Signaling Proteins

A recent identified new family of negative regulators of cytokine signaling is the suppressor of cytokine signaling (SOCS). The SOCS family, including the immediate early gene CIS (cytokine inhibitor of signaling), comprises at least eight proteins (SOCS-1 to SOCS-7, CIS) with similar structures (Hilton et al., 1998; Starr and Hilton, 1999). The SOCS proteins contain a variable region at the N-terminus, a centrally located SH2 domain mediating binding to phosphotyrosine residues in target proteins, and a region of homology at the C-terminus termed the SOCS box. The SOCS box is a region of approximately 40 amino acids which is unrelated to the sequence of other motifs and is of unknown function. Searches on DNA databases for sequences homologous to SOCS box have identified 12 proteins containing this motif (Hilton, et al., 1998). Besides the SOCS proteins, three new families of proteins containing either WD-40 repeats, SPRY domains or ankyrin repeats N-terminal to the SOCS box were described. The function of these proteins is still unknown. Furthermore, a class of small GTPases contains a SOCS box motif. Expression of these proteins exhibit unique tissue-specific and time-dependent responses to a broad range of cytokines, with different stimulation kinetics between the different SOCS genes, that is mediated, at least in part, by STAT proteins. CIS was found to bind to the tyrosine phosphorylated Epo and IL-3 receptors and to block STAT5 phosphorylation and downstream transcriptional responses and, in this way, to suppress proliferation of hematopoietic cells in response to IL-3. In contrast, SOCS-1 binds to the phosphorylated JH1 domain of JAK2 and this association is likely to be mediated, at least in part, by its SH2 domain, although this interaction alone is insufficient for inhibition of a biological response. SOCS/CIS mRNA expression can be induced in liver cells in

response to a pulse of GH, and both SOCS-1 and SOCS-3 were found to inhibit GH-induced STAT5-dependent transcriptional responses in transfected cells (Adams et al., 1998; Favre et al., 1999; Hansen et al., 1999). Expression of other SOCS genes, SOCS-2 and CIS, were also up-regulated by GH, although to a lesser extent than SOCS-3 and with differing kinetics (Adams, et al., 1998). SOCS/CIS proteins inhibit GH-stimulated tyrosine phosphorylation of STAT5b and STAT5b-dependent gene transcription by three distinct mechanisms, distinguished by their targets within the GHR-JAK2 signaling complex. GHR tyrosine residues 333 and 338, required for GH-stimulated lipogenesis and protein synthesis, and phosphorylated in response to GH stimulation, but are not obligatory for GH-stimulated STAT5b activation, are known to play a key role in mediating the inhibitory effects of SOCS-3 on GH signaling (Lobie, et al., 1995; Ram and Waxman, 1999; VanderKuur et al., 1995). The binding of SOCS-3 to GHR tyrosines 333/338 could inhibit JAK2 signaling to STAT5 by interfering with JAK2 binding to the adjacent GHR box-1 region (residues 298-311), thereby raising the Kd of the receptor-kinase complex (Ram and Waxman, 1999). SOCS-1 acts at the level of JAK2 tyrosine kinase, as JAK2 tyrosine phosphorylation is strongly inhibited by SOCS-1 protein (Ram and Waxman, 1999). SOCS-1 inhibition of JAK2 kinase activity requires binding interactions between SOCS-1's SH2 domain and tyrosine 1007 within JAK2's kinase activation loop (Yasukawa et al., 1999). In addition, SOCS-1 can bind to the 80 COOH-terminal cytoplasmic residues of GHR, even in the absence of tyrosine phosphorylation, suggesting that it may be associated with unstimulated receptor molecules. Possibly, the binding of SOCS-1 to these GHR sequences may serve to correctly orient SOCS-1 or perhaps increase its affinity for the receptor-kinase signaling complex (Ram and Waxman, 1999). It has been reported that the SOCS box binds elongins B and C (Kamura et al., 1998). The elongin BC complex has been shown to bind elongin A to form an active transcriptional elongation complex or to the von Hippel Lindau (VHL) tumor suppressor protein (Kaelin and Maher, 1998). The VHL/elongin B-C (VCB) complex also contains a putative E3 ubiquitin ligase (Cullin-2) that may target VHL-binding proteins to destruction by the proteasome (Stebbins et al., 1999). Interestingly, elongin B contains an ubiquitin-like (UBL) sequence at its N-terminus, suggesting a model for the action of the SOCS proteins (Lonergan et al., 1998). First, the

N-terminal and SH2 domains of SOCS-1 and SOCS-3, at least, are required for recognition and binding to activated JAKs. On the other hand, the SOCS box brings elongins B and C into this complex. The model suggests that either through direct interactions of the elongin B UBL domain with the proteasome or through associated Cullin-2-induced ubiquitination of substrates and subsequent proteosomal association, JAK2 and the associated SOCS proteins may be destroyed (Zhang et al., 1999). SOCS-1 was shown to program the hematopoietic specific guanine nucleotide exchange factor, VAV, for ubiquitin-/proteasome-mediated degradation by acting as a substrate-specific recognition component of a VCB-like ubiquitin ligase complex (De Sepulveda et al., 2000). Furthermore, CIS was shown to become ubiquitinated and its degradation was inhibited in presence of proteasome inhibitors upon Epo receptor-stimulation (Verdier et al., 1998). CIS inhibits GHR-JAK2 signaling by two distinct mechanisms: a partial inhibition that is decreased at elevated STAT5b levels and may involve competition between CIS and STAT5b for common GHR cytoplasmic tail phosphotyrosine-binding sites, and by a time-dependent inhibition, that involves proteasome action (Ram and Waxman, 2000). GH was shown to induce degradation of CIS, but not SOCS-3, and the proteasome inhibitor MG132 blocked CIS degradation as well as it inhibitory action on STAT5b signaling (Ram and Waxman, 2000). Proteasome-dependent degradation of CIS, most likely in the form of a (GHR-JAK2)-CIS complex, is therefore proposed to be an important step in the time-dependent CIS inhibition mechanism. The down-regulation of GHR-JAK2 signaling to STAT5b seen in GH-treated cells, as well as for cells treated with Epo and Interleukin-2, could be prevented by treatment with different proteasome inhibitors, suggesting that SOCS proteins are key mediators of cytokine-JAK-STAT desensitization response seen in cells and tissues exposed to the different cytokines (Verdier, et al., 1998). Recently, it was demonstrated that suppression of JAK2 activity was dependent on SOCS-1, where its SOCS box mediated proteasomal degradation of JAK2 rather than JAK2 kinase inhibition (Kamizono et al., 2001). Degradation of JAK2 depended on its phosphorylation and its high-afinity binding with SOCS-1 through the kinase inhibitory region and the SH2 domain. The SOCS box of SOCS-1 was found to interact with the E3 Cullin-2 and promoted ubiquitination of JAK2. This interaction demonstrated the substrate-specific E3 ubiquitin-

ligase-like activity of SOCS-1 for activated JAK2, providing a novel strategy for the suppression of oncogenic tyrosine kinases (Kamizono, et al., 2001).

Ubiquitination of Plasma Membrane Proteins

Several permeases and transporters, like yeast Gap1p, and cell surface receptors, like hormone receptors, are internalized and degraded in a ligand-dependent and ubiquitin-dependent manner (Bonifacino and Weissman, 1998; Hicke, 1999; Hicke and Riezman, 1996; Staub et al., 1997; Strous and Govers, 1999; Strous, et al., 1997). In most cases, the proteasome does not recognize these modified proteins, suggesting that ubiquitination functions in down-regulating such proteins from the cell-surface. Ubiquitination appears to activate the internalization and endocytic machineries that route the internalized proteins to the vacuole/lysosome, where they are finally degraded. Possibly, ligand binding to its receptor triggers either exposure of the ligase binding site(s) or phosphorylation sites. Phosphorylation by the appropriate kinase then recruits the conjugation machinery. The mechanisms by which ubiquitin triggers internalization of membrane proteins are still unknown. Normally, internalization is triggered by tyrosine- or di-leucine motifs, which interact with the clathrin adaptor protein AP-2, resulting in clathrin activation and coated pit formation (Marks et al., 1997; Trowbridge, 1991). These clathrin-coated buds will then pinch off from the plasma membrane and form clathrin-coated vesicles. Analogous to AP-2, ubiquitin could act as an adaptor between lysine residues in membrane proteins and clathrin, or between amino acid sequences in the ubiquitin molecule itself and AP-2 molecules.

In yeast, G protein-coupled α-factor receptors, like Ste2p (Hicke and Riezman, 1996) and Ste3p (Roth and Davis, 1996), and transporters, like uracil permease Fur4 (Galan and Haguenauer-Tsapis, 1997), the maltose permease Mal61 (Lucero and Lagunas, 1997), the galactose transporter Gal2p (Horak and Wolf, 1997), and the ABC peptide transporter Ste6p (Kölling and Hollenberg, 1994), are ubiquitinated upon ligand binding. Ubiquitination of these proteins marks them for proteolysis, resulting in

their internalization and degradation in the vacuole, the yeast equivalent of the lysosome.

Monoubiquitination of certain proteins, like Ste2p and Gal2p, has been shown to be sufficient to trigger endocytosis, suggesting that ubiquitination of such proteins is not involved in proteasomal degradation, since monoubiquitinated proteins are not a target for the proteasome, Fur4p seems to be modified by polyubiquitin chains, but the ubiquitin moieties are linked via Lys63, not Lys48 that is commonly used for ubiquitin attachment, a linkage that appears to be necessary for maximal rates of endocytosis (Galan and Haguenauer-Tsapis, 1997). Since endocytosis of Fur4p requires the "destruction box", it seems likely that ubiquitination motifs necessary for the degradation of certain proteins are also involved in endocytosis of other proteins (Strous and Govers, 1999).

In mammalian cells, it was shown for the Met receptor (Jeffers et al., 1997) and the platelet-derived growth factor (PDGF) receptor (Mori et al., 1995), that ubiquitination might initiate proteasome action. Ubiquitinated forms of these proteins were observed in endosomal/lysosomal compartments (Doherty et al., 1989; Laszlo et al., 1990), suggesting that the proteasome is involved in a partial degradation of the cytosolic tails, while the lumenal and transmembrane domains are degraded in the lysosome. Less clear is the role of the ubiquitin system in endocytosis and degradation of the receptors for immunoglobulin E (Paolini and Kinet, 1993), SLF (Miyazawa et al., 1994), and the T cell antigen receptor (Cenciarelli et al., 1992). Internalization of ENaC depends on ubiquitination of its α- and γ-subunits by the E3 Nedd4 (Schild et al., 1996; Staub et al., 1996), and its rapid turnover is affected by inhibitors of both the proteasome and lysosome. It was suggested that the assembled ENaC complex subunits might be degraded by the proteasome, while unassembled subunits could be degraded by the proteasome. As for the GHR, it was shown that ligand-dependent GHR endocytosis also depends on an intact ubiquitin system (Govers et al., 1997; Strous et al., 1996).

Several steps in the internalization/degradation pathway could be regulated by ubiquitination. In some cases, it is not the receptor, but another non-receptor protein that requires ubiquitination e.g. Eps15, a protein that becomes receptor-associated, phosphorylated, ubiquitinated, and localized to the clathrin-coated pits upon ligand binding of the EGFR (Fazioli et al.,

1993; van Delft et al., 1997a; van Delft et al., 1997b). In other cases, ubiquitination plays a role in the endosomal sorting pathway. Some receptors such as EGF, PDGF and colony stimulating factor (CSF-1) receptors, induce the ubiquitination of a tyrosine kinase regulator, c-cbl (Miyake et al., 1998; Wang, et al., 1996), that participates in the endosomal sorting by binding and ubiquitinating the cytoplasmic domain of the endocytosed receptors (Levkowitz et al., 1998). Such ubiquitinated receptors are localized to the lysosome for degradation, while those that are not ubiquitinated are recycled to the cell surface. The precise role of the proteasome is still unclear. Involvement of proteasomes in the degradation of cytosolic tails has not been established for any of the described proteins. Degradation products in the absence or presence of specific proteasome inhibitors have not been described, not even when antibodies were directed against the extracellular 'proteasome-undegradable' part of these cell surface proteins. This does not exclude proteasome action, as the proteasome might degrade the cytosolic domains of these proteins gradually, during their transport form the plasma membrane to the lysosome. This would result in degradation intermediates of different lengths, which are not easily detectable by immunoblotting (Alves dos Santos, et al., 2001b; Strous and Govers, 1999)

Ubiquitin System-Dependent Endocytosis of the GHR

In the presence of ligand GHR endocytoses rapidly via clathrin-coated pits (Roupas and Herington, 1988), and its degradation occurs at least partially within the lysosome (Murphy and Lazarus, 1984).The GHR was initially found ubiquitinated upon amino acid sequencing of the receptor from rabbit liver (Leung, et al., 1987). The ubiquitin system is required for ligand-induced GHR internalization (Strous, et al., 1996). In a Chinese hamster cell line carrying a temperature-sensitive E1 enzyme (ts20 cells), inactivation of E1 results in accumulation of non-ubiquitinated GHRs at the plasma membrane, while internalization of the transferrin receptor is unaffected (Strous, et al., 1996). In contrast to several other proteins (Staub, et al., 1997; Terrell et al., 1998), ubiquitination itself is not important for endocytosis, as substitution of all the lysine residues in the cytosolic tail did

not affect the process (Govers et al., 1999). Yet, the ubiquitin conjugation machinery is still essential for endocytosis, and a 10 amino acid sequence in the tail, designated ubiquitin endocytosis (UbE) motif and involved in both GHR ubiquitination and ligand-induced endocytosis (Govers, et al., 1999), is required for this activity. Probably, this motif serves, directly or via adaptor proteins, as an anchoring site for possible E2s/E3s, leading to coated pit localization and subsequently to GHR internalization. Upon binding of the ubiquitinating enzymes, interaction with an endocytic adaptor, e.g. AP2 could occur, or alternatively, the E2/E3 complex could serve as an adaptor, analogous to the role of β-arrestin for the β-adrenergic receptor (Goodman et al., 1996). If ubiquitination of a GHR-associated protein is involved in the endocytosis process, possible candidates could be Eps15 and c-Cbl, as mentioned above. The UbE motif does not resemble any other known ubiquitination domain (Govers, et al., 1999), and mutation of the aromatic as well as the acidic residues did not allow internalization. In particular, the phenylalanine at position 327 mutated to an alanine blocked internalization. Possible candidates containing an UbE motif and reported to be also ubiquitinated are for instance, the prolactin receptor (Cahoreau et al., 1994), the PDGF receptor (Mori et al., 1992), and the c-erbB-2 receptor (Mimnaugh et al., 1996).

Recently, the proteasome has been shown to also be involved in GHR down-regulation (Alves dos Santos, et al., 2001b; van Kerkhof et al., 2000). GHR internalization requires proteasomal action in addition to an active ubiquitin conjugation system. Specific proteasomal inhibitors block GH uptake of the full-length GHR, while a truncated receptor can endocytose undisturbed. Furthermore, when the GHR is truncated at position 349, a latent di-leucine endocytosis motif becomes active and the UbE motif is no longer required for ligand-induced internalization (Govers, et al., 1999). This suggests that, upon activation of this di-leucine motif by removing a major portion of the tail, the GHR cytosolic tail is cut from the C-terminus (Alves dos Santos, et al., 2001b; van Kerkhof, et al., 2000). Growth hormone receptor internalization and signal transduction are independently regulated by the ubiquitin system. Mutation of the attachment site for Jak2, box-1, in the GHR cytoplasmic tail resulted in the complete absence of GHR and Jak2 phosphorylation. This modification did not alter the rate and extent of receptor-bound GH internalization as compared to a functional GHR, nor

did it change its turnover and transport to the plasma membrane (Alves dos Santos et al., 2001a).

The Ubiquitin System and the Stress Response

Stresses such as elevated temperatures, heavy metals, amino acid analogs, viral infection, oxidative and chemical damage, or the production of defective proteins trigger vital cellular responses, either to repair the damage, or to limit its toxicity to the cell. Normally, transcriptional induction of a set of genes whose products, known as stress proteins, enhances survival under stress conditions (Reviewed in:Pickart, 1999). The synthesis of heat shock proteins at sub-heat shock temperatures is triggered by the forced production of denatured proteins (Goff and Goldberg, 1985), indicating that protein damage is the key event in the induction of the stress response. When the ubiquitin pathway was described as a mechanism for selective degradation of abnormal proteins containing amino acid analogs, it first confirmed its important role in stress response (Ciechanover et al., 1984; Finley et al., 1984; Hershko et al., 1982). Subsequently, certain components of the pathway were found to be stress proteins, and ubiquitin conjugation is increased in heat shock and other stress conditions. Ubiquitin itself is a stress protein, where its expression is induced by diverse stresses, including heat shock (Finley et al., 1987), heavy metals (arsenicum and cadmium) (Jungmann et al., 1993), amino acid analogs (Finley, et al., 1987), DNA-damaging agents (Treger et al., 1988) and oxidants (Cheng et al., 1994). Imposition of stress is usually accompanied by a dramatic redistribution of ubiquitin, such that most of the ubiquitin in the cell becomes conjugated to substrate proteins. The requirement of ubiquitin expression in stressed cells reflects the need to replenish the pool of free ubiquitin, and in the absence of such replenishment, then the ubiquitination of denatured proteins is inhibited and these species accumulate to toxic levels. Expression of specific E2 enzymes is also up-regulated in stressed cells (Seufert and Jentsch, 1990). In yeast, UBC5 and UBC5 genes are important for the ubiquitination and turnover of normal-short-lived proteins, but also in heat shock and cadmium induced stress (Jungmann, et al., 1993; Seufert and Jentsch, 1990). No E3 enzymes have yet been identified as stress proteins, nor has any E3

been shown to play an important role in the stress response. Stress induces an increase in the levels of ubiquitin conjugates. The fraction of ubiquitin that is unconjugated decreases, while the fraction of ubiquitin in conjugates increases (Finley, et al., 1987; Kulka et al., 1988; Shang and Taylor, 1995). This effect can be extreme: in unstressed cells, about 50% of the ubiquitin is unconjugated (Haas and Bright, 1985), whereas less than 10% of the ubiquitin is unconjugated in heat-shocked HeLa cells (Carlson and Rechsteiner, 1987). The use of synthesis inhibitors like cycloheximide, does not prevent stress-induced ubiquitin redistribution, suggesting that this event is mainly due to increased susceptibility of proteins to ubiquitination. However, up-regulation of ubiquitin and E2s might also contribute to increased conjugate levels (Shang et al., 1997).

Upon oxidative stress, both protein ubiquitination and proteasomal degradation have been demonstrated (Ramanathan et al., 1999; Shang, et al., 1997). Oxidative stress damages cells, and has been implicated in several processes, inculding degenerative diseases of aging, Alzheimer's and Parkinson's disease, arthritis, atherosclerosis, and cancer (Ames et al., 1993; Crawford and Blankenhorn, 1991; Jenner, 1994; Keller et al., 2000; Sziraki et al., 1998; Witztum and Steinberg, 1991). The exposure of cells to high levels of oxygen free radicals results in damage of proteins, lipids, DNA, and enzyme activity, affecting both cell proliferation and differentiation. Proteins containing thiol groups/Cys residues are sensitive targets of oxidants, where, for instance, phosphorylation /dephosphorylation activities are affected. Several proteins involved in signal transduction, including MAPKs, Raf-1, Ras, and growth factors receptors, such as for EGF and PDGF, are phosphorylated and activated upon oxidative stress (Abe et al., 1998; Gonzalez-Rubio et al., 1996; Knebel et al., 1996; Rao, 1996; Rosette and Karin, 1996). It is thought that the increased EGF receptor phosphorylation upon hydrogen peroxide (H_2O_2) exposure, is a result of tyrosine phosphatase inactivation (Knebel, et al., 1996; Lee and Goldberg, 1998). Following cellular exposure to oxidants, the levels of the primary cellular sulfhydryl reductant glutathione (GSH) decrease, while the levels of its oxidized form (GSSG) increase. Upon severe oxidative stress, ubiquitination of proteins is also decreased, suggesting that oxidative stress interferes with E1/E2 activities (Jahngenhodge et al., 1997; Shang and Taylor, 1995). These enzymes contain active site sulfhydryls that might be covalently modified

(thiolated) upon enhancement of GSSG levels (glutathiolation). Glutathiolation might regulate the levels of ubiquitinated proteins in response to oxidative stress (Jahngenhodge, et al., 1997). Furthermore, the 20S proteasome was found more resistant to oxidative stress than the ATP- and ubiquitin-dependent 26S proteasome (Reinheckel et al., 1998).

Ubiquitin-dependent receptor endocytosis is also affected by oxidative stress. Both the chicken hepatic lectin (CHL) receptor, a transmembrane receptor that mediates endocytosis of glycoproteins terminating with N-acetylglucosamine or other glucose-related structures (Chiacchia and Drickamer, 1984), and the EGFR (De Wit et al., 2000) ligand-dependent internalization were shown to become inhibited upon H_2O_2 stress. Possibly, H_2O_2 inhibits EGF receptor internalization by inhibition of Eps15 ubiquitination, known to be involved in the internalization process (De Wit et al., 2001). Whether GHR constitutive levels at the plasma membrane are also affected by stress-induced ubiquitination, is a question that still remains.

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Inhibition of the Ubiquitin-Proteasome System by a Viral Repetitive Sequence

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Introduction

Two decades after the discovery of the ubiquitin/proteasome system, it is hard to name a single cellular function that is not directly or indirectly controlled by this sophisticated proteolytic machinery (Hershko and Ciechanover, 1998). The list of cellular events that rely on ubiquitin-dependent proteolysis include mechanisms as diverse as inactivation of transcription factors (Ashcroft and Vousden, 1999) and cell cycle regulators (DeSalle and Pagano, 2001; Jorgensen and Tyers, 1999), relocalization and degradation of endoplasmic reticulum-resident proteins (Plemper and Wolf, 1999; Wiertz et al., 1997), internalisation of cell-surface receptors (Strous and Govers, 1999) and the release of transmembrane transcription factors (Hoppe et al., 2001). Each of these complex events is initiated by the recognition and ubiquitination of a specific substrate by ubiquitin ligases that interact with highly selective recognition signals in the target protein. The first example of such 'degradation signals' was identified soon after the discovery of ubiquitination as the N-end rule degron according to which the half-life of a protein is determined by its N-terminal amino acid residue (Bachmair et al., 1986). Several natural degradation signals have been subsequently described, including the PEST sequence, the Destruction box and many more (Laney and Hochstrasser, 1999). Degradation signals have two common characteristics. First, they act by recruiting a ubiquitin ligase

which usually results in proteasomal processing of the substrate. Second, they are autonomous modular domains and can be transferred to a different protein, resulting in specific ubiquitination.

Our understanding of degradation signals has increased enormously over the last few years. Yet, it seems unlikely that degradation signals would be the sole determinant of the stability of proteasome substrates. We have previously described a viral sequence that can influence the turnover of protein substrates and, like a degradation signal, functions as transferable autonomous module (Dantuma et al., 2000a; Levitskaya et al., 1995; Levitskaya et al., 1997; Sharipo et al., 1998). This viral sequence slows down proteasomal degradation and appears therefore to counteract the effect of degradation signals. In this review, we will discuss the possibility that this viral sequence might be the first example of a 'stabilization signal' and speculate about the presence of similar stabilization signals in other cellular substrates that can fine-tune or abrogate proteasomal degradation.

The Art of Hiding

The word 'waste' is not of much relevance for the cell's vocabulary as the cellular environment is a paradigm for optimal usage of resources. An elegant illustration is provided by the exploitation of peptide fragments generated by the proteasome as a representative blend of the cell's protein content, which is crucial for triggering cellular immunity in animals with an adaptive immune response (Rock and Goldberg, 1999). Once loaded onto major histocompatibility (MHC) class-I molecules, these peptides are the main information source for cytotoxic T lymphocytes (CTLs) that scrutinize the cell surface in search for signs of modifications caused by infectious agents or any other type of intracellular insult. In their zeal to avoid recognition by the host's defence system, intracellular pathogens have developed an array of strategies to counteract different events involved in antigen presentation (Ploegh, 1998). The Epstein-Barr virus (EBV) has learned to manipulate the proteolytic machinery.

EBV is a lymphotropic γ-herpes virus that causes latent infection in man (Masucci and Ernberg, 1994). A vast majority of the human population carries this virus that usually causes asymptomatic infections but is

unfortunately also associated with epithelial and lymphoid malignancies (Baumforth et al., 1999). Like other viruses that can hide within their host organism, EBV can chose between lytic or latent expression programs. While the lytic program supports the production of viral particles and sacrifices the host cell, in the latent program only a limited number of viral proteins are expressed enabling the virus to remain dormant in resting B lymphocytes or replicate together with its proliferating host cell (Masucci and Ernberg, 1994). At least nine latent viral proteins are expressed in EBV transformed B-cell blasts including six EBV nuclear antigens (EBNA1-6) and three latent membrane proteins (LMP1, LMP2A and LMP2B). Depending on the latency program, different sets of these proteins are expressed in EBV associated malignancies (Masucci and Ernberg, 1994). Immunologic analysis revealed that EBV carriers have CTLs recognizing a number of different epitopes derived from these latent proteins. Since immunosuppression frequently results in EBV-associated lympho-proliferative diseases in EBV carrying hosts (Baumforth et al., 1999), a delicate balance between immune evasion and recognition of EBV infected cells appears to achieve control of the virus without eradication. A puzzling observation emerging from early studies of CTL responses was the failure to isolate CTL clones able to recognize cells expressing the EBV nuclear antigen EBNA1 (Rickinson and Moss, 1997). This was especially intriguing since EBNA1 is the only protein expressed in each of the latency programs (Masucci and Ernberg, 1994), which is probably explained by the primary function of EBNA1 in maintaining the viral episomes in proliferating cells (Leight and Sugden, 2000). The CTL invisibility of an important latent protein would generate obvious advantages for the virus, suggesting that a specific strategy may have evolved to prevent recognition of EBNA1. This justified a closer examination of the EBNA1 protein in relation to antigen presentation.

Several functional domains in EBNA1 are involved in DNA binding, the formation of homodimers and repression of transcription (Leight and Sugden, 2000). Close to its N-terminus EBNA1 contains a mysterious repetitive sequence consisting of solely glycine and alanine residues. Even though this glycine-alanine repeat (GAr) varies in length between different virus strains, it is consistently long ranging from approximately 60 to more then 300 amino acids and can form up to one third of the full sized protein.

In a number of key experiments we showed that the GAr is responsible for the lack of recognition of EBNA1-expressing cells by CTLs (Levitskaya et al., 1995). Cells expressing an EBNA1 chimera carrying a characterized epitope were not recognized by CTLs specific for this epitope confirming that EBNA1 is not subject to antigen presentation. However, when the GAr was deleted from the chimeric EBNA1 the epitope was recognized by CTLs resulting in killing of the cells. Moreover, transfer of the GAr to another viral protein, EBNA4, which is an efficient target for CTLs (Rickinson and Moss, 1997), completely abrogated presentation of EBNA4-derived epitopes (Levitskaya et al., 1995). A subsequent study confirmed these *in vitro* data in a mouse model showing that presentation of EBNA1 can only be accomplished in the absence of the GAr (Mukherjee et al., 1998). Thus the GAr domain blocks selectively the presentation of epitopes from EBNA1. The identification of the GAr as the inhibitor of presentation opened also the possibility to readdress the absence of EBNA1-specific CTLs in EBV carriers. A study in which EBNA1 lacking the GAr was expressed as a target protein revealed surprisingly that EBNA1 specific CTLs are present in healthy carriers but fail to recognize cells expressing full length EBNA1 explaining why they were not noticed in the earlier surveys for EBV-specific CTLs (Blake et al., 1997). These EBNA1-specific CTLs were only able to lyse target cells when the EBNA1 protein was exogenously added suggesting that they originate from cross priming by cells that internalised EBNA1 by endocytosis. The relevance of these CTLs in controlling EBV infection is questionable, as EBV harbouring cells seems to evade recognition.

The path that leads from full sized protein to epitopes at the cell surface is complex, consisting of the generation of small peptides, translocation of the peptides to the endoplasmic reticulum by a transporter complex, loading of the peptides onto MHC class-I molecules and relocation of the MHC class-I-peptide complex to the cell surface (Rock and Goldberg, 1999). Since many of these steps are known to be prime targets for viral evasion strategies, the next step was to identify at what point the GAr interferes with the presentation of EBNA1.

Detailed *in vitro* analysis unmasked a unique mode of action for the GAr. While many viral evasion strategies target the MHC class-I complex (Collins and Baltimore, 1999; Wiertz et al., 1997), the GAr appeared to

interfere with one of the first steps: the generation of peptides by proteasomal processing (Levitskaya et al., 1997). Ubiquitination of GAr-containing chimeras was unaffected but the proteins were nevertheless resistant to proteasomal degradation, an astonishing finding at that point considering that ubiquitination was believed to be the decisive event in ubiquitin-dependent proteolysis.

The GAr is a Transferable Modular Signal

Two important findings inspired us to investigate how universal the inhibitory effect of the GAr might be. First, it was clearly shown that the protective effect on proteasomal degradation and antigen presentation could be transferred to another EBV protein simply by insertion of the GAr (Levitskaya et al., 1995). Second, processing appeared to be blocked downstream of ubiquitination, suggesting that inhibition may occur regardless the involvement of specific degradation signals or ubiquitin ligases (Levitskaya et al., 1997).

To challenge the inhibitory effect of the GAr, its activity was analysed in a number of natural and artificial proteasome substrates each bearing different degradation signals. GAr-containing variants were generated of the inhibitor of NF-κB, IκB-α (Sharipo et al., 1998), the tumor suppressor p53 (Heessen et al., 2002) and two artificial green fluorescent protein (GFP)-based proteasome substrates tagged with N-end rule or ubiquitin fusion degradation (UFD) signals (Dantuma et al., 2000a). Indeed, we observed that proteasomal degradation of each of the substrate was abrogated or at least severely impaired by the introduction of the viral repeat.

The cellular IκB-α was most susceptible to the protective effect as its degradation was completely blocked by an engineered GAr as short as eight amino acids, a length that is far below those found in EBV isolates (Sharipo et al., 1998). Two different ubiquitin/proteasome-dependent pathways degrade IκB-α: a slow constitutive turnover and a fast inducible degradation, for example, in response to TNF-α (Karin and Ben-Neriah, 2000). Both pathways were blocked by insertion of the GAr (Sharipo et al., 1998). Notably, the chimeras were functional and TNF-α stimulation of cells expressing these IκB-α-GAr chimeras induced apoptosis, similar to

what has been observed with other stabilized IκB-α variants. Functional stabilisation was also observed with the tumor suppressor p53 (Heessen et al., 2002). Insertion of the GAr blocked proteasomal degradation mediated by the cellular ubiquitin ligase Mdm2 or the human papiloma virus (HPV) E6/cellular E6-AP complex while preserving p53 potential to induce cell cycle arrest and apoptosis. Indeed the p53-GAr had improved growth inhibitory activity in a HPV^+ cervix carcinoma cell line and an osteosarcoma cell line expressing elevated Mdm2 levels. Using GFP-based proteasome substrates that allow quantification of proteasomal degradation in living cells (Dantuma et al., 2000b), we showed that the GAr also overrides the N-end rule and UFD signals (Dantuma et al., 2000a). Cells expressing the GFP substrates emit reduced fluorescence as compared to cells expressing stable GFP due to rapid destruction of the reporter by the proteasome, while insertion of the GAr resulted in a significant increase in the fluorescence intensities.

The location of the repeat within the host protein seems to be of minor importance since in IκB-α (Sharipo et al., 1998), p53 (Heessen et al., 2002), and the GFP reporters (N.P. Dantuma, unpublished observations), both N-terminal and C-terminal insertion had very similar inhibitory activities. Moreover, in EBNA4 and IκB-α even repeats inserted in the middle of the protein were tested and shown to be protective (Levitskaya et al., 1997; Sharipo et al., 1998). Together these studies show that the GAr functions as an independent transferable module and is active regardless its location in the target protein. Thus, the GAr appears to be a true stabilization signal that can partially counteract or fully override a broad array of degradation signals.

Repeat Composition and Length

A closer examination of the natural GAr sequence reveals that it is an imperfect repeat of single alanine amino acids separated by one to three glycine residues (Baer et al., 1984). Strikingly, the alanine residues are never adjacent to each other and never spaced by more than three glycine residues. To study the constraints of the repeat the IκB-α-GAr chimera were most helpful as short repeats of eight amino acids were sufficient for full

protection from proteasomal degradation. The original protective octamere contained three alanine residues (GGAGAGAG) (Sharipo et al., 1998). Conversion of this to a stretch of eight glycines abrogated the inhibitory effect while eight alanine residues were almost as protective as the consensus repeat (Sharipo et al., 2001). Increasing the spacing between the alanines to four glycine residues resulted in complete loss of the protective effect (Sharipo et al., 2001). Substitution of the alanine residues with another hydrophobic residue, valine, yielded a repeat as active as the original GAr but the inhibition was lost when the alanine residue was replaced with a polar, serine, or charged, aspartic acid, residue (Sharipo et al., 2001). Hence it appears that regularly spaced hydrophobic residues are important for the inhibitory activity.

The protective effect accomplished by a short repeat in IκB-α brings up the question why EBV bothers to carry such a long GAr in EBNA1. The answer to this question came from studies with GFP-based proteasome reporters, which carry degradation signals that target the GFP with different efficiencies (Dantuma et al., 2000b). The degradation of GFP reporters carrying sub-optimal degradation signals was fully blocked by the insertion of a small GAr whereas GFP reporters that were targeted more efficiently were only partly protected by the short repeat (Dantuma et al., 2000a). The effect was strongly improved using a full sized GAr from a natural EBV isolate although even then residual proteasomal degradation was observed. Increasing the levels of the specific ubiquitin ligase can enhance the proteasomal degradation. In accordance, the effect of increasing the levels of either Mdm2 or HPV E6 could be countered by inserting longer GAr in p53-GAr chimera (Heessen et al., 2002).

In summary, studies with different proteasome substrates show that the strength of the GAr-mediated stabilization signal is determined by the length and composition of the repetitive sequence and by the presence of optimally spaced hydrophobic residues. The GAr can counteract several unrelated degradation signals and its effect is inversely proportional to the strength of the degradation signal or the amount of ubiquitin ligase. Yet, the unanticipated susceptibility of IκB-α, which despite its short half-life is fully protected by a very short GAr, indicates that other factors may strongly influence the effect of this stabilization signal.

Mode of Action

The unique inhibitory activity of the GAr brings up the obvious question as to the mechanism by which this is accomplished. Collectively, the available evidence suggests that a step in between ubiquitination and proteasomal degradation is probably affected. Unfortunately, very little is known about this segment of the pathway.

The proteasome is one of the most sophisticated machinery in the cell, which reflects its involvement in complex events such as substrate recruitment, extraction of substrates from protein complexes or membranes, substrate unfolding, tethering of the substrate into the proteolytic cavity, substrate hydrolysis and release of the peptide fragments into the cytosol (Voges et al., 1999). The GAr could interfere with this process by introducing structural changes that render the substrate resistant to the unfoldase activity of the proteasome. Notably, the only known example of a long repetitive GAr in nature is silk which consists of a perfect alternating repeat of glycine alanine residues forming an antiparallel β-sheet structure (Lotz and Keith, 1971). Silk is appreciated for its stability and it is tempting to speculate that the EBV GAr may form a similar robust structure. Indeed, recent data support the idea that local protein stability can be an important determinant in artificial substrates (Lee et al., 2001). However, structural analysis of an IκB-α chimera bearing the octameric GAr using mass spectroscopy and circular dichroism revealed that the repeat is unstructured in the context of IκB-α (Leonchiks et al., 1998). This does not exclude the possibility that the GAr may assume a stable conformation upon encounter with a specific binding partner. However, additional data argue against the formation of an indigestible structure. In particular, the residual proteasomal degradation of highly destabilized GFP variants, even when a GAr of 239 amino acids was inserted, demonstrates that the proteasome is physically capable of degrading the repeat (Dantuma et al., 2000a). In line with this observation, it was shown that EBNA1 itself can be degraded by the proteasome once provided with an efficient degradation signal (Dantuma et al., 2000a). This results also in the generation of antigenic peptides of EBNA1 and recognition by CTLs (Tellam et al., 2001). Thus, the proteasome can degrade GAr containing proteins but this requires very efficient targeting for degradation.

An alternative explanation for the inhibitory effect is that the GAr modifies the interaction between the regulatory 19S complex of the proteasome and the substrate. Indeed, the interaction between an unfolded non-ubiquitinated protein and the 19S regulatory complex results in refolding and release (Braun et al., 1999). We found that a polyubiquitinated p53-GAr chimera was still able to interact with the S5a polyubiquitin-binding subunit of the regulatory complex suggesting that presence of the GAr does not preclude binding to the proteasome (Heessen et al., 2002). However, while polyubiquitinated IκB-α could be co-immunoprecipated with the proteasome complex, a polyubiquitinated IκB-α-GAr was, under the same conditions, predominantly free of both its binding partner NF-κB and the proteasome (Sharipo et al., 1998). Hence, it is tempting to speculate that the GAr may modulate the interaction between the polyubiquitinated substrate and the 19S regulatory complex resulting in more transient interaction that is less likely to be followed by proteolytic processing.

Other Stabilization Signals

Viral pathogenicity and immunoescape strategies are often an exaggerations of cellular processes, which explain why some critical cellular functions were originally discovered through the study of viruses. Functional analogues of the EBV GAr have been found in related herpes viruses infecting baboons and rhesus monkeys although these related repeats do not fully block presentation of epitopes derived from the corresponding viral proteins (Blake et al., 1999). Yet, the question remains whether cellular proteins harbour stabilization signals that may block or fine-tune their turnover. Such signals may be hard to find by sequence comparison, as the consensus sequence is poorly defined. Nevertheless, there are a few interesting candidates although conclusive evidence for each of these proteins is still lacking.

Processing of p105. The NF-κB member p105 is targeted for ubiquitin/proteasome-dependent proteolysis but, unlike other substrates, p105 is not entirely destroyed and proteolysis is limited to the C-terminal half of the protein, resulting in the generation of p50 (Karin and Ben-Neriah, 2000). Processing is precisely regulated and depends on an internal signal

that determines the point at which the proteasome releases the p50 product. This internal signal contains multiple glycine residues and is often referred to as the glycine rich region (Lin and Ghosh, 1996). Interestingly, two alanine residues are also present in this stop signal, giving rise to a GAGAG sequence (Orian et al., 1999). A recent study probed into the importance of these two alanine residues in the processing of p105 and provided evidence that the GAGAG sequence is a major determinant (Orian et al., 1999). The authors postulated that, similar to the GAr, these alanine residues block proteasomal degradation. This is an interesting possibility that strengthens our model of interference with a late event, most likely during the interaction of the polyubiquitinated substrate and the 19S regulatory subunit.

Polyglutamine repeats. Several neurodegenerative disorders, including Huntington's disease, Kennedy's disease and different ataxias, are caused by expansion of a polyglutamine tract in proteins (Zoghbi and Orr, 2000). Interestingly, these diseases are characterized by the presence of insoluble nuclear inclusions consisting of the expanded polyglutamine repeat-containing protein and various components of the ubiquitin/proteasome system (Cummings et al., 1998). It has been postulated that the expanded polyglutamine repeats renders the target protein resistant to proteasomal degradation resulting in accumulation and aggregation into insoluble protein complexes. Indeed it has been shown that one of these proteins, ataxin-1, is a true substrate for the ubiquitin/proteasome system and that expansion of the repeat coincides with a threefold decrease of proteasomal degradation *in vitro* (Cummings et al., 1999). Similarly, the N-terminal fragment of huntingtin with expanded polyglutamine repeats was shown to be more resistant to proteasomal degradation than its counterpart with a non-pathologic repeat (Jana et al., 2001). Although these recent data are of potential interest in relation to the GAr, it should be emphasized that cells expression GFP-GAr fusions failed to form protein aggregates (Dantuma et al., 2000a), a characteristic that is probably critical for the effect of the glutamine repeat.

Polyalanine repeats. Our analysis of the constraints on repeat composition revealed that long stretches of alanine residues were almost as protective as the original GAr consensus. Interestingly, expansion of polyalanine stretches in core binding factor α1 (Mundlos et al., 1997), HoxD13 (Muragaki et al., 1996), polyalanine binding protein-2 (Brais et al.,

1998), and Zic2 (Brown et al., 1998) have been reported and these mutant proteins are linked to developmental disorders known as cleidocranial dysplasia, synpolyldactyl, oculapharyngeal muscular dystrophy and holoprosencephaly, respectively. For one of those disorders it was shown that the severity of the congenital malformation correlates with the length of the repeat (Goodman et al., 1997). Conceivably, alanine tracts could modify the stability of proteasome substrates in a length dependent manner. Although a proteasome-dependent regulation of these proteins is not unlikely in view of their spatial and temporal expression patterns, ubiquitin-dependent proteolysis has only been shown for core binding factor $\alpha 1$ whose turnover appears to be tightly regulated and of major importance for osteoblast development (Tintut et al., 1999). An intriguing possibility is that the alanine expansions may cause the corresponding disorders by inducing locally and temporally elevated levels of the target proteins.

Concluding Remarks

The twists and turns of EBV that enable this virus to survive and flourish in the hostile environment of the human body have disclosed a novel type of regulatory element: the stabilization signal. The idea that the turnover of many cellular proteins may be regulated through a balance of degradation and stabilization signals is intellectually pleasing. However, many questions remain still unresolved and more efforts should be devoted to the mode of action of this inhibitory signal and the identification of functionally or structurally related sequences in other protein substrates. The virus has put us on the track of this new regulatory domain but elucidation of its mechanism reaches far beyond the boundaries of EBV biology. The solution to this riddle could provide us with important clues on the activity of the ubiquitin/proteasome system.

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Autosomal Recessive Juvenile Parkinsonism and the Ubiquitin Pathway

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Abstract

Autosomal recessive juvenile parkinsonism (AR-JP) is the most prevalent form of familial Parkinson's disease (PD). AR-JP is characterized by selective and exhaustive loss of dopaminergic neurons in the substantia nigra of the midbrain. Clinical features of AR-JP resemble those of idiopathic PD, but AR-JP lacks Lewy bodies, the pathological hallmark of idiopathic PD. *Parkin*, the causative gene of AR-JP, encodes a 52-kDa protein that is a RING-finger type ubiquitin (Ub)-protein ligase (E3) collaborating with an Ub-conjugating enzyme (E2) belonging to a cognate class of UbcH7 or UbcH8. Analysis of parkin mutations in AP-JP patients reveals that the functional loss of parkin as an E3 enzyme is the molecular basis of AR-JP, suggesting that accumulation of as-yet-unidentified protein(s) causes nigral neuron death. Thus, it is clear that abnormalities of proteolysis mediated by the Ub-proteasome pathway are involved in AR-JP pathogenesis. These findings should shed new light on the mechanisms underlying neurodegeneration in idiopathic PD as well as AR-JP.

Introduction

To date, it becomes clear that surprisingly over 30% of newly-synthesized proteins are discarded without being properly folded (Schubert et al 2000). In addition, even if proteins are normally formed into tertiary structures, they are continuously impaired by spontaneous denaturation, chemical modification (e.g., oxidation), and environmental stresses, such as increased temperatures, reactive oxygen species (ROS, e.g., free radicals), or heavy metals (especially mercury) (Sherman & Goldberg 2001). In rare cases, dysfunctional proteins with highly abnormal conformations result from genetic mutations. In considering these circumstances, it is expected that a rubbish heap of abnormal proteins accumulate in the cells. However, none of them are detected in normal healthy cells, because of the elaborate system that prevents accumulation of these improperly folded proteins or abnormal proteins generated in the cells. For this purpose, two mechanistically antagonistic strategies have been acquired during evolution. One is the chaperone system that prevents the aggregation of the unfolded molecules and helps to refold them, e.g., by a Hsp90- or Hsp70-assisted fashion (Sherman & Goldberg 2001). The other is called the ubiquitin (Ub)-proteasome system (Coux et al 1996; Hershko & Ciechanover 1998; Hershko et al 2000; Voges et al 1999). When severely damaged proteins cannot be refolded into their functional forms, cells hydrolyze the abnormal proteins to small polypeptides by this protein-destroying machinery (see below) and ultimately back to amino acids by oligo- and exopeptidases.

Ubiquitin is a highly conserved small protein present universally in eukaryotic cells, which is covalently attached to target proteins via an isopeptide linkage between the C-terminal Gly of Ub and the ε-NH_2 group of Lys residue of the acceptor substrate by a cascade system, consisting of activating (E1), conjugating (E2), and/or ligating (E3) enzymes (Hershko & Ciechanover 1998; Hershko et al 2000). Since E3 enzymes, called also Ub-protein ligases, bind to specific protein substrates, they presumably play an important role in the selection of proteins for degradation. In successive reactions, a poly-Ub chain is synthesized by progressive transfer of Ub moieties to Lys-48 of the previously attached Ub molecule. The resultant poly-Ub chain acts as a degradation signal for proteolytic attack by the 26S

proteasome, a eukaryotic ATP-dependent multi-protease complex (Coux et al 1996; Voges et al 1999).

The Ub-proteasome pathway is capable of controlling the level of cellular proteins rapidly, timely, and irreversibly and is advantageous for regulation of divergent protein functions in eukaryotic cells. Indeed, this proteolysis plays a pivotal role in the control of a diverse array of cellular activities, such as cell-cycle progression, signal transduction, immune response, and developmental program (Hershko et al 2000). In addition, the proteolytic pathway is also responsible for selective destruction of proteins misfolded in a protein biosynthetic pathway or unfolded by postsynthetic damage (Sherman & Goldberg 2001). These impaired proteins may be partially unfolded, which are promptly ubiquitinated and degraded by the 26S proteasome. Therefore, we provisionally defined the Ub-protein ligase(s) catalyzing ubiquitination of these unfolded or misfolded proteins as "quality-controlling E3". Notably, the Ub-proteasome pathway plays a central role in impaired protein-clearance by facilitating their proteolytic removal to maintain normal cell functions. In this context, a recently-identified presumptive E3 ligase, named CHIP with a U-box structure that resembles a RING-finger domain, is quite interesting, because it is associated with Hsp90 and Hsp70 and capable of ubiquitinating unfolded proteins trapped by these chaperones (McClellan & Frydman 2001). CHIP is a molecule responsible for the mechanistic link between refolding and degradation of impaired proteins. It can be regarded as a typical example of the quality-controlling E3.

Certain molecular chaperones are known as heat-shock proteins, indicating that cells need a large amount of these molecules in response to environmental stresses to cope with stress-induced malfunctional proteins. Analogously, excessive stress may cause accumulation of abnormal proteins which exceeds the capacity of the Ub-proteasome system. To respond to such crisis, all eukaryotes have at least one or two poly-Ub genes. Among multiple Ub genes, the poly-Ub gene encodes a tandemly repeated Ub structure, indicating that this poly-Ub gene has been elegantly acquired for efficient production of Ub without repeating transcription and translation (Hershko et al 2000). Moreover, the poly-Ub gene belongs to a family of "heat-shock genes". Obviously, the existence of stress-inducible poly-Ub gene indicates that cells also need a large amount of the Ub pool in response

to environmental stresses, which acts to prevent accumulation of abnormal proteins in cells by selective proteasomal elimination after facilitated ubiquitination of these unnecessary proteins, because accumulation of unfolded proteins can have very deleterious effects on cell function.

There is accumulating evidence for the importance of the Ub-proteasome system in maintaining cell homeostasis. The Ub-proteasome pathway has been recently highlighted in the non-dividing cells of the brain, since neuronal intracellular inclusions are composed of Ub-positive protein aggregates that have been described as a common ultrastructural feature of most neurodegenerative diseases (Floyd & Hamilton 1999; Mayer et al 1998). The typical examples are cytosolic Lewy bodies (LB) in Parkinson's disease (PD), neurofibrillary tangle containing tau in Alzheimer disease, and nuclear polyglutamine aggregates in CAG-repeat diseases. The frequent appearance of Ub-positive intracellular inclusions in many neurodegenerative disorders indicates failure of protein quality control mediated by the Ub-proteasome system.

However, there is no direct evidence showing a functional link between Ub-directed proteolysis and neurodegeneration. Recently, we demonstrated that the abnormality of the Ub-metabolic pathway causes autosomal recessive juvenile parkinsonism (AR-JP), one inherited form of PDs. In this review, therefore, we focus on the role of the Ub-proteasome pathway with a special reference to the nigral neurodegeneration of AR-JP and PD.

Parkinson's Disease and AR-JP

Parkinson's disease is the second most frequent neurodegenerative disorder after Alzheimer disease, which usually occurs in the elderly, affecting approximately 1% of the population above the age of 65, but its etiology remains to be uncovered. PD is a slowly progressive neurodegenerative disease with typical clinical features, such as resting tremor, muscular rigidity, bradykinesia (slow movement), and postural instability (disturbance of balance), with sustained response to levodopa, a biosynthetic precursor of dopamine (Lang & Lozano 1998). The pathological changes include a progressive loss of dopamine neurons in the substantia nigra (SN) pars compacta and locus coeruleus, leading to a deficiency or at least attenuation

of dopamine supplies. The prominent cytopathological hallmark of idiopathic PD is the presence of LB, which are intracytoplasmic inclusions consisting of insoluble protein aggregates and consist of a dense granular core surrounded by a halo of radiating filaments, in the surviving neurons detected at postmortem examination (Pollanen et al 1993).

Clinically defined PD represents a heterogeneous disorder that encompasses a small proportion of individuals with inherited disease and a larger population with seemingly idiopathic disease (Kitada et al 2000). The familial PD forms approximately 5% of all PDs, but seems important to solve the mechanistic cause of dopaminergic neuronal death in not only rare forms of familial-linked PD but also idiopathic PD. So far, five genetic loci have been mapped: *PARK1* (4q21-23), *PARK2* (6q25.2-27), *PARK3* (2p-13), *PARK4* (4p14-16.3), and *PARK6* (1p35-36) (Farrer et al 1999; Gasser et al 1998; Kitada et al 1998; Polymeropoulos et al 1997; Valente et al 2001), although several additional loci have been identified without naming those genes. Of these, *PARK1*, *PARK3*, and *PARK4* encode the causative genes for autosomal dominant forms of PD, while *PARK2* and *PARK6* are related to autosomal recessive early-onset parkinsonism. At present, three independent genes have been implicated in the onset and progression of PD. The *PARK1* gene encodes α-synuclein, which was first discovered as the causative gene of the dominant form of familial PD (Polymeropoulos et al 1997) and recently the *parkin* gene (*PARK2*) was identified as the second familial PD gene whose product named "parkin" (Kitada et al 1998). Furthermore, a mutation (I93M) in the gene encoding Ub carboxy-terminal hydrolase L1 (UCH-L1) has been identified in one family with inherited form of PD (Leroy et al 1998). Of note, α-synuclein, a major component of LB, has been implicated in the pathogenesis of autosomal dominant PD, but they seem to account for cases in only a few families having two mutations (A53T and A30P). In contrast, a variety of mutations in the *parkin/PARK2* gene including missense, nonsense, deletion, and frame-shift mutations have recently been identified in world-wide families with inherited juvenile parkinsonism (Abbas et al 1999; Hattori et al 1998; Lucking et al 2000). Clinical features of AR-JP were first reported by Yamamura in 1973 (Yamamura et al 1973). AR-JP patients present the classical parkinsonian symptoms: tremor, rigidity and bradykinesia. Dystonia of the lower limbs and hyperreflexia are frequent among AR-JP patients, although most clinical

features of AR-JP are indistinguishable from those of patients with idiopathic PD. AR-JP is characterized by selective loss of dopaminergic neurons in the SN and the lack of LB, which are often observed in sporadic PD. The age of onset is usually below 40 years and as early as 6 years (on average when patients are in their 20s).

Parkin: The Causative Gene of AR-JP Encoding an E3 Ubiquitin-Ligase

Parkin whose gene contains 12 exons spanning about 1.5 Mb is a 52-kDa protein that harbors the N-terminal Ub-like (UbL) domain, the C-terminal RING box, consisting of two RING-finger motifs and an in-between RING (IBR), and the unique parkin domain (UPD) which connects two regions, i.e., UbL and RING box (Kitada et al 1998; Shimura et al 2000; Tanaka et al 2001). Of various Ub-conjugating enzymes (E2s), UbcH7 or UbcH8 with a high homology to UbcH7 is preferentially recruited to parkin. The high molecular-mass ubiquitinated proteins are co-immunoprecipitated with parkin when the human dopaminergic neuroblastoma SH-SY5Y cells are treated with the proteasome inhibitor MG132. Mutant parkins from AP-JP patients with nonsense and missense mutations in the RING-box are defective with regard to their ability to associate with UbcH7 and ubiquitinated cellular proteins, whereas deletion or a missense mutation of the UbL or the UPD region results in loss of interaction with ubiquitinated proteins without affecting UbcH7 recruitment. Thus parkin consists of two functionally distinct segments: a UbL/UPD domain responsible for target recognition and a RING-box recruiting E2s for ubiquitination (Shimura et al 2000; Tanaka et al 2001). The discovery of parkin's function as an E3 in the Ub pathway has enhanced our understanding of the molecular mechanisms underlying idiophathic PD as well as AR-JP (see below), and suggests that dysfunction of the Ub-proteasome pathway plays an essential role in the nigral degeneration of the disease.

It is important to note the fairly ubiquitous distribution of parkin in the brain and other tissues as well (Huynh et al 2000; Kitada et al 1998; Shimura et al 1999; Solano et al 2000; Wang et al 2001), in considering why

mutation of parkin causes AR-JP (see below). In addition, the *parkin* gene is highly conserved across species, not only in vertebrates, such as humans, rats, mice but also in invertebrates such as *Caenorhabditis elegans* and *Drosophila melanogaster* (Kahle et al 2000). These findings indicate that parkin may play a common role in the control of metabolic stability of multiple proteins in various organisms.

How Does Loss-of-Function of Parkin Cause AR-JP

Functional analysis of parkin as an E3 suggests that AR-JP is primarily caused by abnormality of cellular protein metabolism due to dysfunction of parkin and there is putative substrate(s) for parkin in dopaminergic neurons. It is also clear that loss-of-function of parkin owing to mutations causes accumulation of the target(s) that is not ubiquitinated and that reduced proteasomal elimination of this parkin substrate(s) might ultimately cause death of dopaminergic neurons in AR-JP. However, there are still several unanswered questions regarding our understanding of the etiology of AR-JP. 1) Why does loss-of-function of parkin cause selective nigral degeneration despite fairly wide spread expression of parkin in not only other regions of the brain but also many other tissues, such as skeletal muscle, heart, and testis? 2) Why do parkin mutations cause early-onset? 3) Why are LB absent in AR-JP? Studies that provide replies to these questions should lead to the identification of the pathogenic process of AR-JP and of other PDs as well. However, the mechanisms leading to AR-JP following loss of function of parkin remain unknown at present. Based on the current available information, we propose two plausible models for onset of AR-JP and the frameworks encompassing both models are depicted in Figs. 1 and 2 (for details, see our recent review: Tanaka et al 2001).

In model-1 (Fig. 1), which provides the mechanism of AR-JP, cumulative increases of a putative parkin substrate(s), provisionally termed X(s), which has yet to be identified, cause selective neuronal death. In this model, the target X protein is simply considered as a factor whose accumulation directly mediates death of dopaminergic neurons; i.e., abnormal accumulation of X forcibly evokes cell death. To explain that genetic defect of the *parkin* gene causes specifically dopaminergic neuronal

death in the midbrain of AR-JP patients without affecting the viability of other cells where parkin is normally expressed, it is hypothesized that expression of parkin target X functioning as a cell-death factor is limited in the pigmented neurons in the brain stem, i.e., the SN and locus coeruleus. According to this model, parkin constitutively degrades substrate X under physiological conditions, indicating that parkin may act as a key regulator of putative mediator X involved in the cell death-signaling pathway.

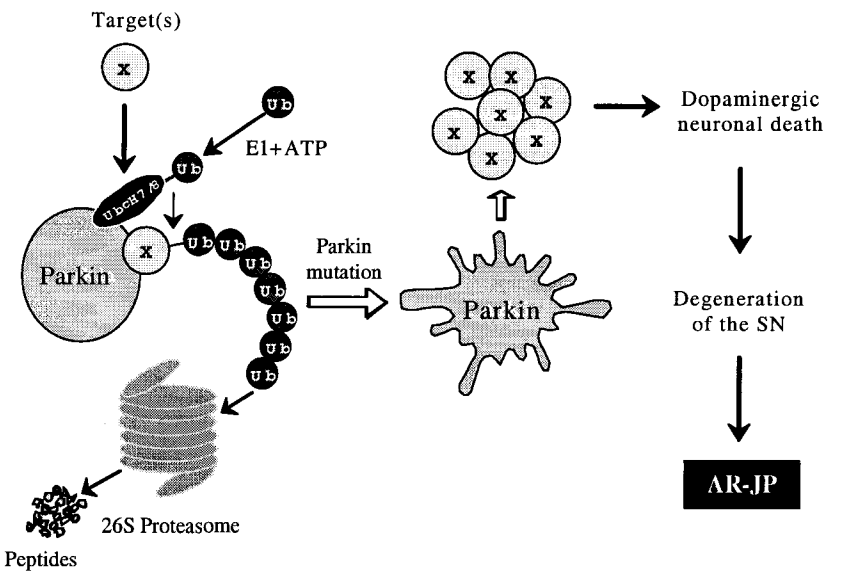

Figure 1. Model-1. Loss-of-function of parkin specifically causes AR-JP. Ub, ubiquitin; SN, substantia nigra; AR-JP, autosomal recessive juvenile parkinsonism. X(s) is a SN-specific substrate(s) whose accumulation in non-ubiquitinated form could activate directly the cell death pathway.

Physiologically, X may contribute to the removal of dopaminergic neuronal cells, when they are accidentally impaired. If so, why does parkin exist in other types of cells and tissues? Probably, in these cells parkin targets a different substrate(s), termed here "Y(s)", which is distinct from X for cell death-signalling. In addition, another unknown Ub-protein ligase(s) may be responsible for ubiquitinating Y and thus in AR-JP no appreciable defects

are observed in these cells due to the compensatory function of this other E3(s), except dopaminergic neurons, in which X is assumed to be mainly ubiquitinated by parkin. The possibility that another E3 ubiquitinates X cannot be excluded, but if true, such activity would be very weak. Alternatively, it is also possible that the level of other E3 is quite low in the SN, differing from other tissues, which is insufficient to ubiquitinate X for degradation without parkin.

In model-1, it is easy to understand why the disease has an early-onset; AR-JP has a germ line mutation of the *parkin* gene, which causes aggressive accumulation of target X, leading to rapid dopaminergic neuronal death. In this case, when the level of X becomes high, the cells die; otherwise it is thought that the cells remain healthy until over the threshold for cell death. Therefore, even though AR-JP occurs during early childhood, appearance of parkinsonism needs many years, e.g., at least 6 years. In addition, as AR-JP mechanistically differs from the LB-forming PD, it is natural that LB are not formed in AR-JP. Alternatively, with regard to the lack of LB in AR-JP, it is also possible that parkin targets a presumptive protein that plays a critical role in LB formation, and the protein may be X or other protein(s). The essence of model-1 is that parkin is directly linked to AR-JP, but not other PDs.

The second model assumes that the cause of AR-JP is mechanistically related to that of sporadic PD, namely parkin is linked to sporadic PD, as illustrated in Fig. 2. In model-2, it is not necessary to predict the existence of a specific substrate X for parkin in the SN. In this model, we hypothesize a two-step mechanism, which is based on our understanding of carcinogenesis. It is generally accepted that tumorigenesis consists of two independent but sequential processes *in vivo*, i.e., 'initiation' and 'promotion'. The 'initiation' step primarily evokes cell-cycle progression, e.g., mostly genetic defects, while the 'promotion' step is a progressive state for cell multiplication and autonomous growth. Analogously, PD could be divided into two distinct processes, i.e., 'initiation' and 'promotion', to understand its pathogenesis. However, definition of these two terms in the onset of PD differs from carcinogenesis as discussed below, because whereas carcinogenesis is linked to unregulated cell proliferation, neurodegenerative cell death is induced in the case of PD. We call tentatively this mechanism as "initiation-promotion hypothesis" in PD.

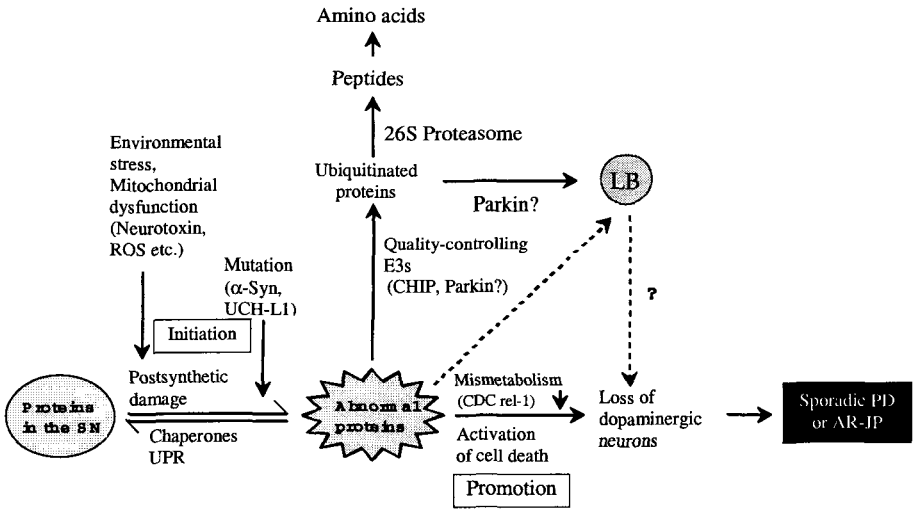

Figure 2. Model-2. Parkin is linked to sporadic PD as well as AR-JP. α-Syn, α-synuclein. For details including environmental stress, neurotoxin, ROS, quality-controlling E3, UCH-L1, CHIP, UPR, CDCrel-1, 'initiation', and 'promotion', see text. In this model, impairment of protein degradation pathway downstream of ubiquitination results in accumulation of ubiquitinated protein(s) and non-ubiquitinated protein(s), i.e., aggregate formation (LB). At present, it is unknown whether parkin is involved in LB formation or LB causes dopaminergic neuron death, however it is currently clear that LB formation is not directly linked to neuronal death and pathogenesis of PD as shown by a dotted line (see text). Note that AR-JP can also be explained by model-2, if parkin operates as a quality-controlling E3.

The 'initiation' in the pathogenesis of PD is provisionally defined as a primary defect(s) of dopaminergic neurons, e.g., environmental factors that cause stresses in the SN. For examples, increased lipid peroxidation, oxidative DNA damage, and protein peroxidation due to ROS (e.g., H_2O_2, OH, O_2^-) and/or mitochondrial dysfunction have been implicated to cause damage of dopaminergic neurons (Beal 2001; Mizuno et al 1998). Indeed, there is accumulating evidence for a decrease in activity of complex I of the mitochondrial electron transport chain in the SN of PD. This defect is site-specific, as it does not occur in other brain areas and it has not been observed in other neurodegenerative diseases. Furthermore, neurotoxins such as 1-methy-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and its active metabolite

metabolite 1-methyl-4-phenylpyridium (MPP^+), rotenone, and 6-hydroxydopamine seem to play prominent roles in the death of dopaminergic neurons: such nigral neurotoxins can be candidates for an initiator of PD. In addition, mutations of α-synuclein and UCH-L1 may induce aggressively the 'initiation' process and thus it is likely that they show autosomal dominant diseases. Presumably, both environmental stress(es) and mutation(s) including the above two genes or other unknown genes are thought to be "risk" factors capable of inducing the nigral neurodegeneration of PD. Therefore, multiple "risk" factors may cooperatively cause PD or a single "risk" factor may be sufficient to cause the disease, when it is severely deleterious in the SN.

The major role of the 'initiation' process is presumably to induce protein damage, i.e., unfolding to highly aberrant conformations, but molecular chaperones may abrogate such abnormal accumulation of impaired proteins by neutralizing them to proper conformation through re-folding of damaged proteins, only when they are not severely impaired. Moreover, as shown in model-2, most of these impaired proteins are rapidly degraded by the Ub-proteasome system under normal conditions. We hypothesize the existence of an E3 Ub-protein ligase(s) responsible for ubiquitination of these partially-unfolded proteins that cannot be refolded, termed provisionally "quality-controlling E3". For instance, CHIP may be a possible candidate, because it binds to chaperones such as Hsp90 and Hsp70 linked with unfolded proteins for ubiquitination, as mentioned above. Thus, quality-controlling E3(s) is thought to play an important role in abnormal protein accumulation and parkin may belong to an E3 enzyme of this category (see model-2). Perhaps, different cells may contain distinct "quality-controlling E3(s)" and parkin contributes importantly in the SN to this function.

When, however, protein damage is induced by stresses and/or changes of conformation due to protein modification or gene mutation, which does not overwhelm the chaperoning and proteolytic delivery systems, the resulting proteins with aberrant structures gradually accumulate in cells. That is, when unfolded proteins cannot refold or be destroyed and accumulate over many years, they become occasionally harmful, induce dysfunctional states, and ultimately cause death of dopaminergic cells. We here call this process as the 'promotion' step in the pathogenesis of PD. As a

consequence of aggressive 'promotion', a large proportion of dead SN dopaminergic neurons consequently causes sporadic PD. In other word, PD can be regarded as one case of "so-called protein-storage diseases" and excess accumulation of deleterious protein(s) can induce the death of dopaminergic neurons. However, at present it is unknown whether the dopaminergic neuronal death occurs by apoptosis or by other types of cell death.

In general, the ultimate fate of stress-induced aberrant proteins will be determined by a balance between re-folding and destruction in cells. However, under the presence of genetic defects of the Ub-proteasome system components or severe injury of cellular proteins, such impaired proteins may escape destruction, indicating failure in the surveillance system for abnormal protein or protein quality control. In this regard, degradation by the 26S proteasome requires almost complete unfolding of substrate proteins to be entrapped and their transport into the chamber where the proteolytic active sites are located (Coux et al 1996; Voges et al 1999). Polyubiquitination itself is required for trapping targets, but not for protein unfolding. However, if denaturation occurs insufficiently or genetic defects continuously produce proteins with highly aberrant structure, these proteins may accumulate in the cells as ubiquitinated or non-ubiquitinated forms without being destroyed by the 26S proteasome. They may aggregate and ultimately form inclusion bodies, such as LB in idiopathic PD. However, whether LB are the cause of neuronal death in PD or consequence of a protective event against cell death, or represents an unrelated event, has not yet been determined.

As noted, the formation of LB is a landmark process in the pathology of PD, although the exact process of the formation of these inclusion bodies remains unclear. In this context, it is worth noting that parkin immunoreactivity is present in LB of sporadic PD (Shimura et al 1999), although a recent study challenged this view (Huynh et al 2000). Thus, it is also possible that parkin is responsible for the formation of LB. If true, it may explain why LB are absent in AR-JP. According to model-2, mutation of parkin increases accumulation of abnormal proteins, and reaches early the threshold level necessary to cause death of neuronal cells. In other words, loss of parkin function accelerates the 'promotion' step and thus AR-JP

occurs as an early-onset disease. Therefore, AR-JP is thought to be one alternative type of PD, which is caused by dysfunction of parkin.

It was recently reported that parkin is linked to unfolded protein response (UPR) in the endoplasmic reticulum and overexpression of parkin suppresses UPR-induced cell death that has been implicated recently (Imai et al 2000). In this point of view, the 'promotion' in PD may be linked to the signalling pathway of UPR by unknown mechanism. It was also found that parkin targets CDCrel-1, a septin family protein, which is predominantly expressed in the nervous system for ubiquitination (Zhang et al 2000). It is interesting that parkin and CDCrel-1 are localized in synaptic vesicle membranes (Kubo et al 2001; Zhang et al 2000), indicating that CDCrel-1 is a strong candidate for a natural parkin substrate. As CDCrel-1 binds to syntaxin and inhibits exocytosis, mutation of parkin may induce mismetabolism in the cells caused by abnormality of exocytosis due to excess accumulation of CDCrel-1, which also influences the 'promotion' process resulting in pigmented cell death in the midbrain. In addition, it must be emphasized that 'initiation' or 'promotion' process itself could probably induce PD, if each occurs heavily in dopaminergic neurons.

Perspective

In this review, we proposed two models in AR-JP: model-1 for the function of parkin whose dysfunction causes AR-JP (Fig. 1) and model-2, which hypothesizes that AR-JP is an alternative form of sporadic PD (Fig. 2). In model-1, we predict that parkin target X is a factor whose accumulation can activate the death-signalling pathway, which is only present in dopaminergic neurons, as a sequence to reduced E3-function of parkin. In contrast, in model-2, we predict that parkin is a member of the quality-controlling E3 family and is involved in the LB formation. At present, we do not know which model is correct, but we favor the latter scenario shown in model-2, because the presence of SN-specific substrate X seems unlikely and it is plausible that parkin is present in LB. However, to date many neuropathologists follow the former model-1 and believe that AR-JP differs from sporadic PD based on pathological evidence, i.e., LB must be detected at postmortem examination in the surviving neurons of PD.

To date, several studies have indicated that environmental stresses, such as ROS, may influence proteasome function in the central nervous system. If proteasome dysfunction specifically occurs in dopaminergic neurons, then the possibility that stress-dependent vulnerability of proteasomes ceases mandatory destruction of ubiquitinated proteins and influences the onset of PD can not be ruled out completely. However, these observations appear to be superficial so far and this point of view is only just beginning to emerge. Therefore, we think such paradigms are beyond the scope of this review and await future comprehensive analysis.

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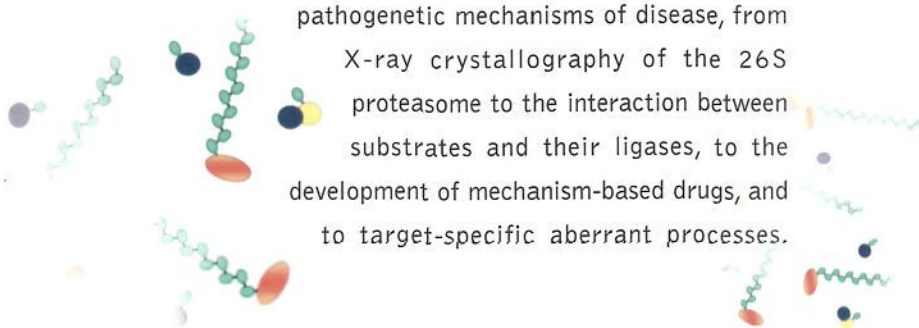

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