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ROLES OF UBIQUITINYLATION IN PROTEOLYSIS AND CELLULAR REGULATION

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ABSTRACT

Most eukaryotic organisms respond to starvation, nutrient deprivation, and/or stress by increasing the rates of intracellular proteolysis. The amino acids released may be reutilized for synthesis of important proteins, or directly for the production of energy. This enhanced proteolysis is also required for repair of cellular damage due to environmental insults such as heat shock, free radicals, viral infection, or mutation. Finally, intracellular proteolysis is important in determining the steady-state levels of a wide variety of regulatory proteins, particularly those regulating the cell cycle. The ubiquitin-dependent proteolytic system participates in all of these functions. In spite of its cytoplasmic localization, this system is selective and acts only on a limited set of substrates. This review discusses the mechanisms of this selectivity and the potential roles of ubiquitin-dependent proteolysis.

INTRODUCTION

The classic response to starvation, nutrient deprivation, and/or stress includes an increased rate of intracellular proteolysis. At least three general types of proteolytic reactions have been implicated: proteolysis by soluble or membrane-bound proteases [often responding to second signals such as Ca^{2+} (25)], upregulation of the lysosomal proteolytic machinery upon carbon starvation (29), and stimulation of the ubiquitin-dependent proteolysis system (60). The focus of this review is the latter system, which has been shown to respond to glucocorticoids during fasting (133), to tumor necrosis factor (TNF) [which may increase in cachexia (44, 85)], to metabolic acidosis (93), to γ-interferon elicited by viral infection (1, 10), to feeding cycles (70), to heat shock (39), and to damaged proteins (3). Because several general reviews on this subject have recently appeared (21, 48, 60, 65, 74, 106, 108, 126), I limit references to the most recent or comprehensive contributions.

Ubiquitin is a small, highly conserved protein present universally in eukaryotic cells. It functions by covalently attaching to other proteins, resulting in the targeting of those proteins for specific cellular fates. The covalent linkage is an isopeptide bond between the C-terminus of glycine 76 (gly76) of ubiquitin and the side-chain amino groups of lysine on the target protein. Ubiquitin can be thought of as a posttranslational signal sequence that is attached to a variety of cellular proteins and that can target proteins to different fates depending on the context of the environment. The ultimate effects of ubiquitinylation will depend on the protein to which it is attached as well as on the localization of the conjugates and complex enzymatic specificities. Only a few of these effects are understood, and only protein degradation has been studied in any detail.

This review describes this proteolytic role and speculates on the other possible roles of ubiquitinylation.

All cellular proteolytic systems must be regulated in response to intracellular signals, many of which are ill defined. Both the lysosomal and the ubiquitin-dependent systems function as compartmentalized multienzyme systems that sequester proteolytic sites from the bulk of soluble proteins. The lysosome packages its proteases in an acidic compartment and acquires substrates by either autophagy or selective uptake through the hsp73 receptor (29). The ubiquitin-dependent system sequesters its proteolytic sites by including them in a multienzyme complex and selects substrates by ubiquitinylation of the target protein. This posttranslational covalent modification commits the target proteins to degradation.

The overall process of ubiquitinylation involves four separate reactions: activation of ubiquitin, which requires the expenditure of one adenosine triphosphate (ATP) molecule per ubiquitin; conjugation of ubiquitin to a variety of cellular proteins; proofreading of the conjugates to either regenerate the target protein by removal of ubiquitin or commit the target protein to proteolysis by adding more ubiquitin molecules; and finally, proteolysis of the conjugates by the proteasome or the lysosome. Through these reactions, proteins are marked by ubiquitinylation and targeted for degradation in the cell. The energy contributed by ATP is utilized to exert specificity on the proteolysis step by specifically marking only certain proteins and delivering them to the proteolytic systems.

At first glance, ubiquitin-dependent processes seem overly complicated, involving well over 100 gene products. However, such complexity enables the ubiquitin-dependent system to play a role in "garbage collection" of damaged proteins in the cell and in the degradation of short-lived regulatory proteins. This system is also involved in the structure and function of receptor-mediated and membrane-bound events, although its role in these processes is less well defined. In general, every step in the pathway is catalyzed by a family of isozymes. In some cases, the specificity of individual isozymes is known to differ and the expression of different isozymes is spatially and developmentally regulated. This variation enables the system to perform a number of functions in different tissues at different stages of development, differentiation, and adaptation.

FORMATION OF POLYMERIC UBIQUITIN

Structure and Chemistry of Ubiquitin

Ubiquitin is a highly conserved, 76-residue protein universally present in eukaryotic cells. Because the structural properties of ubiquitin have been

described previously (129), only a few points are elaborated here. The three-dimensional structure of ubiquitin shows that the site of protein attachment, the C-terminus, protrudes from the globular body of the protein. Ubiquitin is a very stable protein that refolds rapidly. It is also stable to most proteases at neutral pH. These properties are probably advantageous since ubiquitin participates in the delivery of substrate proteins to proteases. Second, ubiquitin undergoes a well-characterized conformational change in a hydrophobic environment. As discussed below, this conformational change may be important in the "proofreading" of protein structure (24).

Structure of Polymeric Ubiquitin

The chemistry of ubiquitin is dominated by the formation and cleavage of peptide bonds at its carboxyl-terminus (Figure 1). In all species studied, ubiquitin is coded for by three classes of genes (75). Class I and II genes encode two proteins, each consisting of a fusion protein between ubiquitin and a zinc-finger protein. These carboxyl extension proteins are proteolytically processed to yield ubiquitin and the zinc-finger proteins (Figure 1), ribosomal proteins required for efficient ribosome biogenesis (37). Thus, transcription from class I and II genes results in fusion proteins that are processed into two halves: one that is required for ubiquitin-dependent protein degradation and another that is necessary for protein synthesis. These genes are expressed constitutively in rapidly growing cells and may consequently coordinate rates of protein synthesis and protein degradation. The class III gene encodes a ubiquitin precursor consisting of head-to-tail repeats of the ubiquitin protein sequence. The number of repeats varies with the organism, but this transcript is strongly induced by stress and is regulated by a consensus heat-shock promoter. The proprotein synthesized from this gene must also be proteolytically processed to release monomeric ubiquitin.

In addition to these gene products, a second type of ubiquitin C-terminal derivative is formed when one or more ubiquitins are added to target proteins. The best characterized of these conjugates is formed by the attachment of the C-terminus of ubiquitin to the side-chain amino group of lys-48 of another ubiquitin (50). Long chains of this type form on target proteins and must be disassembled during or after proteolysis of the target protein. These polymeric ubiquitin molecules are linked by ε-amino amide bonds and are sufficient to target proteins for degradation (50). In some cases, other lysines on ubiquitin may be involved in polyubiquitinylation, although the consequences of these alternative linkages are not understood.

Enzymes of Ubiquitin Conjugation

The covalent attachment of ubiquitin targets proteins for specific cellular fates. Proteins can be mono-, di-, or polyubiquitinylated. In all cases, the bond is

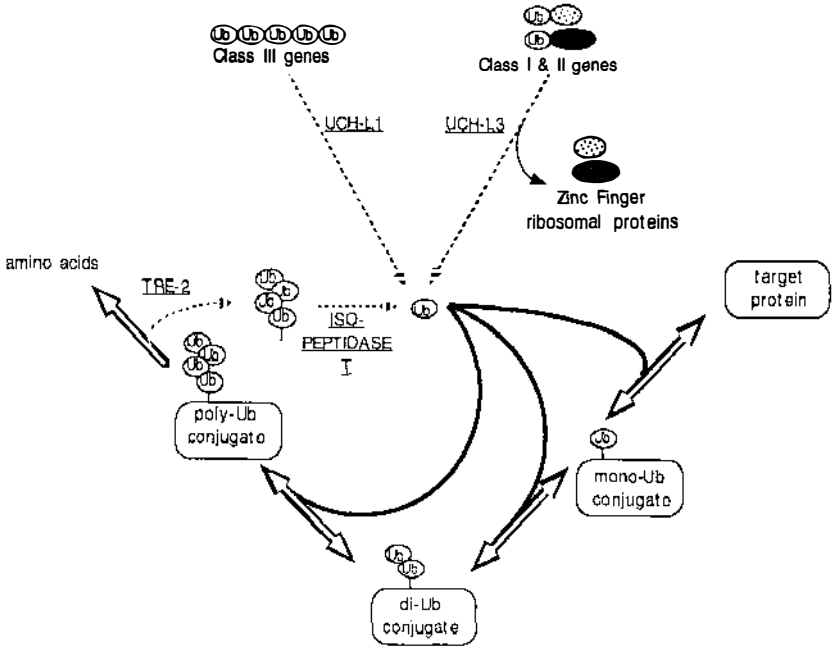

Figure 1 Metabolism of ubiquitin. Dashed arrows indicate processing reactions catalyzed by ubiquitin carboxyl-terminal hydrolases or by ubiquitin-specific proteases. Solid arrows represent reactions of conjugation and proofreading. Open arrows represent the path for targeting and degrading cellular proteins.

formed between an amino group of the target protein (usually the side chain of lysine) and the carboxyl-terminus of ubiquitin. The specificity of ubiquitinylation is not understood, but it involves a complex series of reactions catalyzed by the first three enzymes of the system.

E1: THE UBIQUITIN-ACTIVATING ENZYME The first enzyme, the ubiquitin-activating enzyme, catalyzes the formation of a thiol ester between the C-terminus of ubiquitin and a cysteine residue of the ubiquitin carrier proteins. The subcellular localization depends on the cell cycle (51), and ubiquitin activation is probably important in a variety of cellular sites at different stages of the cell cycle.

Much of what is known about the function and biological roles of ubiquitin-dependent processes has been discerned from the phenotype of conditional lethal mutations in the ubiquitin-activating enzyme. A surprising proportion of conditional lethals with defects in cell-cycle progression are ts mutants in this enzyme (60, 75). Synchronized cultures of these cells arrest in S and S/G2

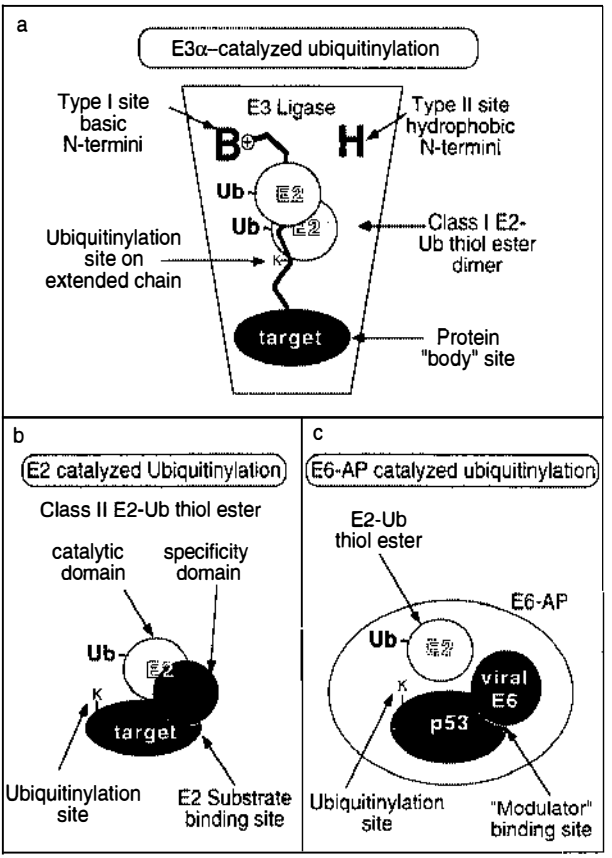

Figure 2 Enzymes involved in the conjugation of ubiquitin to cellular proteins. (*a*) The E3 ubiquitin protein ligase responsible for conjugation to the N–end rule substrates utilizing class I E2 carrier proteins. (*b*) Class II E2 carrier protein–catalyzed conjugation to basic proteins. (*c*) The E6-AP/E6-catalyzed ubiquitinylation of p53. This is an example of other possible conjugation routes.

upon shifting to the nonpermissive temperature. They fail to activate ubiquitin or condense chromatin and exhibit reduced degradation of abnormal and short-lived proteins (38).

E2: UBIQUITIN CARRIER PROTEIN(S) After ubiquitin is activated by thiol ester formation with E1, it undergoes *trans*-thiol esterification with a cysteine residue on one of the E2 proteins. E2 genes, including at least 10 separate genes in yeast, have now been characterized in many organisms (75). All share a central core of approximately 150 residues that are 35% identical and that

contain the catalytic thiol. Class I enzymes consist of the core only and require E3 (ligase) in order to conjugate ubiquitin to target proteins (Figure 2*a*). Class II enzymes have C-terminal extensions, which are often highly charged. These extensions are required for direct ubiquitinylation and are thought to be involved in substrate recognition (80, 96). Class III enzymes have both C- and N-terminal extensions, and the N-terminal domains are also important in substrate selection (76, 127). Such extensions may also play a role in protein-protein interactions, in membrane anchoring, or in binding to specific E3 ligases.

In the case of the small E2 proteins, the activated ubiquitin is delivered as the E2 thiol ester to the E3 ligase protein complex for conjugation to the target protein (105). These small E2s include the ubiquiting-conjugating enzymes UBC4 and UBC5 in yeast (75), the E2 (14k) from reticulocytes (105), and a 15-kDa wheat E2 (45). The specificity of this conjugation pathway depends on the specificity of the E3 ligase(s) as well as on the identity of the E2. These small E2 proteins have been termed ubiquitin carrier proteins because of their mechanistic similarity to fatty acid activation. Some of the larger E2s, however, can conjugate ubiquitin directly to target proteins (105) or to ubiquitin (16) and are thus referred to as ubiquitin-conjugating enzymes.

E3: UBIQUITIN PROTEIN LIGASE(S) Some ubiquitinylation reactions require the recognition of a particular protein or structure by a binding protein, followed by association with a ubiquitin carrier protein core domain and by ubiquitinylation of the target protein. An enzyme thought to be involved in this recognition and catalysis was first identified as a large-molecular-weight component of the ubiquitin pathway that is necessary for ubiquitinylation and subsequent degradation of proteins in the reticulocyte extracts (60). This enzyme, termed E3 ligase, has affinity for substrate proteins and must bind E2 (14K), at least transiently, in order to transfer the ubiquitin from this carrier protein to the target protein. Genetic and biochemical evidence (60, 126) suggest that binding is also specific for the nature of the amino terminus of the protein. A site specific for a basic residue at the N-terminus and a second site specific for a large hydrophobic residue at the N-terminus of the target protein are apparently present on the same polypeptide (called E3α). Dipeptide esters are useful inhibitors of this enzyme (60). Proteins with basic amino termini bind to the basic site, as shown in Figure 2*a*. This binding is inhibited by basic dipeptide esters. Conversely, proteins with large hydrophobic amino termini bind in the second site and are prevented from binding by the corresponding dipeptide esters. This specificity is the basis of the finding that the nature of the amino-terminal residue in a protein sequence can determine the half-life of the protein (6, 7, 126). Thus, it was observed that simply varying the amino-terminal residue caused the half-life of β-galactosidase constructs to range from 2 min to >20 h. This principle was termed the N–end rule and is thought to be the

manifestation of the enzymatic specificity of E3α. The yeast enzyme, encoded by the UBR1 locus, has been cloned and sequenced (9). It encodes a protein of 1950 residues with a slightly acidic isoelectric point and a molecular weight of approximately 225,000. Null mutants of UBR1 are viable, do not degrade N–end rule substrates, and exhibit partial defects in sporulation and growth.

The fact that UBR1 mutants are not lethal suggests that other systems may conjugate ubiquitin for ATP-dependent proteolysis. A second reticulocyte ligase, E3β, has been described (59). This protein shares many functional and physical properties with E3α but recognizes substrates with A, S, or T as the amino-terminal residue. Another yeast factor (E3-R) appears to cooperate with the RAD6 gene product (UBC2) in catalyzing the monoubiquitinylation of several proteins otherwise not ubiquitinylated by this E2 (113). Finally, a recently cloned protein, E6-AP, binds to the complex of papilloma virus E6 and p53, resulting in its ubiquitinylation and subsequent degradation (71; Figure 2*c*). In this reaction, E6-AP is an E3 that recognizes specific complexes between viral and cellular proteins and results in the ubiquitinylation and degradation of the conjugate.

PROPROTEIN PROCESSING AND DISASSEMBLY OF POLYUBIQUITIN CHAINS

The above discussion details the formation of ubiquitinylated proteins. Equally important is the processing of the various polymeric forms of ubiquitin. A number of steps in ubiquitin metabolism require proteolytic processing by cleavage of amide bonds at the C-terminus of ubiquitin (Figure 1). In general, these processing enzymes are thiol proteases with specific recognition sites for ubiquitin and the protein C-terminal to ubiquitin. The cleavage must occur at gly76 of ubiquitin in order to accurately process these polymers. Two types of enzymes with this specificity have been described: the ubiquitin carboxyl-terminal hydrolases, which are thiol proteases of approximately 25,000 daltons (132); and ubiquitin-specific proteases, a group of thiol proteases of between 100 and 150 kDa (8, 121).

Proofreading of Protein Ubiquitinylation

In principle, the aforementioned enzymes can stimulate or inhibit ubiquitin-dependent processes. Inhibition could result from the deconjugation of ubiquitin from either mono- or polyubiquitinylated protein substrates. These reactions would reverse the conjugation of ubiquitin and define a futile cycle. We have postulated that this futile cycle is a proofreading mechanism designed to assure that only damaged proteins become marked and degraded by this system (24). This hypothesis suggests that ubiquitin is conjugated to most proteins. If the protein is stable, proteases are present that will recognize the

native conformation of ubiquitin and remove it. If, however, the protein is damaged, unstable, or denatured, the exposed hydrophobicity could cause a conformational change in ubiquitin. It is this altered conformation that is presumably recognized by the committed step in the pathway, i.e. polyubiquitinylation.

Proteolytic Processing of Polyubiquitins

Alternatively, the action of these processing enzymes may stimulate ubiquitin-dependent processes by increasing the steady-state level of free ubiquitin and by the hydrolysis of polyubiquitin chains. Accumulation of the latter would be expected to inhibit proteolysis, since these chains bind tightly to the 26S proteasome (see below). Enzymes with this catalytic activity (hydrolysis of esters or amides at the C-terminus of ubiquitin) are also required for the processing of all ubiquitin gene products and metabolites, including adventitiously formed adducts between ubiquitin and small cellular nucleophiles. Clearly, it is important to understand which enzymes catalyze these reactions and to determine their distribution and regulation.

Ubiquitin Carboxyl-Terminal Hydrolases

Ubiquitin carboxyl-terminal hydrolases (UCH) are a class of small cytoplasmic thiol proteases with specificity for cleavage of small esters and amides at the C-terminus of ubiquitin.

THE UCH GENE FAMILY We have identified and characterized four UCH isozymes from bovine thymus. Using antibodies to one of these proteins, we cloned and sequenced a human cDNA for the major thymus isozyme, UCH-L3 (132). The UCH-L3 sequence exhibits considerable similarity to a yeast protein with the same catalytic activity, to a newly identified *Drosophila* protein prominent in nurse cells (135), and to human PGP 9.5 (UCH-L1), a neuronal-specific protein. These enzymes are thiol proteases unrelated to any other known thiol proteases. The sequences are conserved as strongly across species as they are among isozymes (approximately 40% identity), suggesting that evolutionary pressure to maintain these active structures throughout development is significant.

REGULATION OF UCH GENE EXPRESSION Using both immunological and biochemical approaches (131), we have shown considerable tissue specificity in the distribution of UCH isozymes L1 and L3. Isozyme L1 is strongly expressed in neuronal, neuroendocrine, and perhaps some fetal cells. Isozyme L3 is present mainly in hematopoietic cells. Many tissues and cells contain significant amounts of isozyme L2, which may be a constitutive isozyme. The expression of UCH isozymes is also developmentally regulated, although the

levels of expression have been difficult to modulate in tissue culture (131). The appearance of UCH-L1 (PGP 9.5) immunoreactivity in developing mouse brain correlates with the arrival of the neuronal precursor cells at the neural plate and the elaboration of neural processes (INM Day & R Thompson, private communication). It is induced in the gonads of fish undergoing the sexual transition from female to male (41), in several neural inclusion bodies (89), and in experimentally induced axonal dystrophy (5). Its level is strongly downregulated upon viral transformation of lung fibroblasts (68). Similarly, in *Drosophila,* the mRNA for uch-D is strongly expressed in the nurse cell, the ovary, and the testis (135). The transcripts are also easily identified during the first few hours of development. Within 4–6 h of development, however, the levels of transcript drop markedly. This decrease is consistent with the pattern usually seen for maternal transcripts. Thus, the expression of these genes is temporally and spatially regulated.

PHYSIOLOGICAL ROLE OF UCH ISOZYMES We have hypothesized that the neuronal UCH (UCH-L1) is responsible for the cotranslational processing of the proubiquitin gene product induced by stress response (131). Hemopoietic cells, on the other hand, have less need for this type of stress response but must process the ubiquitin zinc-finger gene products in order to rapidly synthesize the ribosomes needed for these cells to undergo rapid clonal expansion. Thus, the B-cell enzyme (UCH-L3) may be involved in the cotranslational processing of these proteins in rapidly dividing cells. Isozyme L2 is more widely distributed and may play a role in removing ubiquitin from peptides generated as degradation intermediates or in salvaging ubiquitin adventitiously trapped by reaction with cellular amines and thiols.

Recent data (130) confirm that the protease and peptidase specificity of these enzymes is consistent with their role in cotranslational processing. When the leaving group at the C-terminus of ubiquitin is a large folded domain, these enzymes will not hydrolyze the amide bond, perhaps because of steric exclusion from the active site. In cotranslational processing, the substrate is presumably generated after the N-terminal copy of ubiquitin has emerged from the ribosome and folded but before the subsequent peptide sequence can fold into a stable structure.

The role of the *Drosophila* UCH isozyme in development is highly speculative. The uch-D transcripts are preferentially localized to the ventral surface of both the oocyte and the nurse cell. Both dorsal/ventral and anterior/posterior gradients of mRNA and protein accumulation are required for proper embryogenesis in *Drosophila.* One of the mechanisms for establishing this polarity may be the formation of a spatial gradient of proteolytic potential. Indeed, the proteasome is present in such gradients during *Xenopus* development (110). The expression of the ubiquitin-dependent proteolysis system along a concen-

tration gradient in the cell may contribute to the establishment of a gradient of several proteins. Relevant ubiquitin-dependent proteolytic substrates may be those involved in regulation of cell division, signal transduction, cell-cell contact, hormone response, or gene regulation.

Ubiquitin-Specific Proteases

Another class of enzymes catalyzing the cleavage of peptide bonds at the C-terminus of ubiquitin has been characterized from yeast (8, 121). Three genes were identified based on their ability to hydrolyze large ubiquitin-protein fusions: ubiquitin binding protein 1 (UBP1, 809 amino acids); UBP2, 1264 amino acids; and UBP3, 912 amino acids. These proteins are only distantly related, exhibiting homology in limited regions around presumed catalytic residues. UBP1 and UPB2 were generally able to hydrolyze the amide bond between ubiquitin and β-galactosidase, between ubiquitin and dihydrofolate reductase, and between ubiquitin and the natural ribosomal zinc-finger proteins. The precise role of these proteins is unknown, as yeast with interruptions of all three activities fails to exhibit a phenotype.

Interruption of a fourth UBP coding region (DOA4) has revealed a UBP putatively associated with the 26S proteasome (103). This activity releases the polyubiquitin chains from the remnants of proteins degraded by the proteasome. Mutants in this enzyme are defective in the degradation of MAT-α2, the yeast mating factor. This enzyme strongly resembles a human open reading frame, TRE-2, which has transforming activity and may function as an oncogene or a tumor suppressor. The TRE-2 protein contains the UBP active site cysteine but has deleted the active site histidine and is enzymatically inactive (103). Interestingly, mutation of the active site cysteine of DOA4 results in a dominant negative phenotype and accumulation of ubiquitin chains that may have small peptides attached. The accumulation of these chains presumably inhibits the 26S proteasome and subsequently results in general protein degradation (see Figure 1). Thus, the putative cause of this oncogenic transformation are DOA4 homologues, which can bind to the proteasome but are inactive, resulting in the reduced turnover of important regulators of cell-cycle progression. These regulators may include substrates for ubiquitin-dependent proteolysis such as cyclins, c-*mos,* p53, c-*myc,* c-*fos,* c-*jun,* and NF-κB (see below).

After the degradation of ubiquitinylated protein and the release of polyubiquitin chains from the degradation remnant (peptide), the chains must be degraded to release free ubiquitin and to prevent accumulation of inhibitory concentrations of free polyubiquitin chains. One such activity, catalyzed by a 98-kDa protein termed isopeptidase T, has been reported to stimulate protein degradation (56), presumably because isopeptidase T can partially disassemble polyubiquitin chains, thereby increasing the supply of ubiquitin or relieving inhibition caused by the accumulation of such chains. We recently found that

this enzyme efficiently disassembles polyubiquitin chains. It acts by an exo-hydrolytic mechanism, removing one subunit at a time from the C-terminal-most end of the chains (KD Wilkinson & C Pickart, unpublished results). This activity requires an intact C-terminal copy of ubiquitin, and deleting gly-76 or replacing it with an ala residue decreases the rate of reaction by at least three orders of magnitude. The activity is not precessive, but triubiquitin binds more tightly than diubiquitin, which accords with earlier suggestions that ubiquitin has multiple binding sites (56). These results imply that the polyubiquitin chain must be removed from the protein substrate, or peptidyl remnant, before disassembly (Figure 1). This mechanism could prevent such hydrolytic activity from removing the signal sequence prematurely. In addition, it assures that only intact ubiquitin is liberated from these chains for reutilization.

PROTEOLYSIS OF UBIQUITIN CONJUGATES

Once target proteins have been marked for degradation by the attachment of the polyubiquitin, they must be delivered to the proteolytic machinery for degradation (60). The participation of a multienzyme complex (the proteasome) and/or the lysosome (see below) emphasizes the fact that these degradation processes are segregated in the cell. At least two possiblities could account for this complexity: (*a*) The presence of a marking event and a translocation of the substrate to a degradative site enable specificity to be maintained and degradation to be limited to specific protein substrates; and (*b*) catalyzing the degradation in a localized environment rich in proteolytic activities prevents release of degradative fragments (perhaps with significant biological activities).

The Proteasome

In the early 1980s, Rechsteiner and Hershko and their respective colleagues demonstrated that the specific proteases of the ubiquitin-dependent system in reticulocytes were large-molecular-weight complexes that apparently required ATP for both assembly and degradation (48, 60, 108). This active protease had a sedimentation coefficient of 26S and exhibited ATP dependence and selectivity for ubiquitinylated substrates (69). In addition, these authors noted a second form of proteolytic activity that sedimented at 20S and contained many of the same polypeptides. The peptidase activity of this complex was activated by detergents and was not ATP dependent or selective for ubiquitinylated proteins. The term prosome is used here to refer to the 20S multicatalytic proteinase. The term proteasome is reserved for the 26S particle responsible for ubiquitin-dependent protein degradation. The 20S prosome is converted to the 26S proteasome by association with other regulatory proteins, possibly including PA28 (17), PA700 (18), and CF-2 (54).

THE 20S PROSOME The 20S complex contains a characteristic set of 14 polypeptides ranging from 20 to 34 kDa and assembles into a stack of 4 rings, with sevenfold symmetry in the individual rings (4, 49). The resultant barrel-shaped structure exhibits several peptidohydrolase activities on artificial substrates and various proteins, including a trypsin-like, a chymotrypsin-like, and a peptidylglutamyl-hydrolyzing activity.

Many of the subunits of the 20S complex have been cloned and sequenced (118), and more unpublished sequences have been detected in database searches. There is considerable sequence similarity between subunits and across species. The subunits are encoded by two gene families related to the corresponding α and β subunits of the homologous complex in archaebacteria (26). Subunits of the β family occupy the central two disks of the four-disk structure and may contain the active sites necessary for proteolytic activity. These subunits exhibit no obvious sequence similarity to other proteases, and the assignment of proteolytic activities to individual subunits using antibodies, chemical modification, and genetic methods is just beginning (57, 63). Deletion mutants of some of these subunits in yeast are lethal, suggesting an important role for this particle. The α family subunits occupy the outer two disks of the 20S protease. These are the sites of binding of accessory proteins that regulate proteolytic activity (49).

The subunit composition and intracellular localization of the 20S complex appear to vary with the cell cycle (2) and developmental stage in *Drosophila* (55, 78) and *Xenopus* (110), with the proliferative rate in kidney cancer (77), or upon stimulation with γ-interferon (1, 10, 40, 107). The differential subunit composition allows the chromatographic separation of different populations of prosomes (36), although the functional differences are unclear. One 31-kDa subunit has been reported to be localized in prosomes present in the apical domain of hepatocytes (12). This observation suggests some regiospecific function for a subpopulation of prosomes. Expression of specific isoforms may alter the specificity of the 20S complex in certain tissues or developmental states. Two messages encoding Hs PROS-30, differing only in the 5′ untranslated region and the first six amino acids encoded, have been described (114). Thus, at least one of the prosome subunits can be regulated by alternative splicing, a common regulatory mechanism involved in tissue-specific expression. Posttranslational mechanisms may also help regulate the activity or half-lives of subunits. At least two of the subunits have potential phosphorylation sites that may be involved in regulation, and phosphorylation of an ~30-kDa subunit has been reported (60). In addition, an inhibitor of the 26S proteasome (δ-ALA dehydratase) can also be ubiquitinylated (54).

The variability in subunit composition probably plays a role in altering the activities, specificity, or localization of the 20S complex. Some reports have defined this complex as an mRNP particle or associated it with polysomes,

whereas others have claimed that it contains a 90-nucleotide RNA, a tRNA-lys, and/or a pre-tRNA 5′-processing endonuclease activity (100, 108). The 20S prosome has also been implicated in the regulation of translation because it has been found to be inhibitory in reticulocyte extracts (81). These results indicate that this "core protease" may have multiple functions that could be regulated by association of various accessory proteins and that the assembly of the active proteasome may be similarly regulated. A variety of endogenous inhibitors and specificity factors were recently described that have been implicated in the assembly and regulation of the active 26S proteasome (17, 18, 54, 108).

THE 26S PROTEASOME A unifying hypothesis holds that the 20S multicatalytic proteinase is "decorated" with regulatory proteins to yield the active 26S proteasome. This specific case derives from the general hypothesis (60) that the prosome is a degradative particle whose specificity is controlled by its localization, by the composition of its variable subunits, and by the binding of accessory proteins. In order to definitively test this hypothesis, we need to define the accessory proteins that must "decorate" the prosome to convert it to a proteasome as well as the mechanisms and regulation of such an interconversion.

Consider the role of the accessory proteins that bind to the prosome. Three protein fractions and ATP must be recombined to form the 26S proteasome, and the assembly involves a time lag. These protein fractions have been termed CF-1, CF-2, and CF-3. CF-1 and CF-2 were shown to have ATP binding sites, and CF-3 was later determined to be the 20S prosome (60). Association of CF-1 and CF-2 with the 20S prosome is required to confer ATP dependence and specificity for ubiquitinylated proteins. CF-2 was recently identified as δ-ALA synthetase (54), and its binding results in the inhibition of the peptidase and proteinase activities of the 20S prosome (33). CF-1 may be related to a complex (PA700) containing approximately 16 additional polypeptides. That these accessory proteins bind to the ends of the barrel, like the activator PA28, seems reasonable (49). These proteins include subunit 5, which contains the specific binding site for polyubiquitin chains (28) and is probably necessary to bring the substrate into the vicinity of the degradative machinery; one or more ATP-dependent translocators (67), which could require energy to unfold the protein and translocate it to the site(s) of proteolysis; and a ubiquitin-specific protease similar to DOA4 (103), which may remove the polyubiquitin chain from partially degraded proteins.

The Lysosome

The lysosome is another compartmentalized degradative system. It is implicated in the enhanced protein degradation seen in starvation or serum depri-

vation (29) and in nonspecific proteolysis. The uptake of proteins by the lysosome has traditionally been attributed to autophagy. Recently, however, a protein uptake system was described that exhibits selectivity for proteins containing a KFERQ sequence motif. Uptake of proteins containing these sequences is mediated by binding to hsc73, the 73-kDa heat-shock cognate protein (30, 119). Up to 30% of the cytoplasmic protein can be subjected to this enhanced degradation rate. Although this is likely a general nonspecific stress response, significant selectivity to lysosomal degradation is possible.

Obvious parallels can be drawn between proteasomal and lysosomal degradation. A signal sequence (ubiquitin or the KFERQ domain) is recognized by a specific binding protein, and the bound protein is delivered to a degradative environment for proteolysis. Despite this analogy, ubiquitin was only recently determined to be enriched in the lysosome (60). Ubiquitin-protein conjugates accumulate in the lysosome when lysosomal degradation is inhibited and are enriched approximately 12-fold in the presence or absence of lysosomal inhibitors (82). A second line of evidence supporting the role of the lysosome in degradation of ubiquitinylated proteins is the finding that cells with a thermolabile ubiquitin-activating enzyme fail to increase lysosomal proteolysis in response to heat shock (52). Lenk et al have suggested that some ubiquitin-dependent step occurs in the maturation (but not the formation) of autophagic vacuoles (84).

Ubiquitinylated proteins can probably be degraded by both soluble (26S proteasomes) and vesicular (lysosomal) proteases. Michalke et al (90) proposed that the latter is the mechanism of degradation when the ubiquitinylated protein is insoluble, membrane bound, or associated in some way with the cytoskeleton. However, other investigators question the specificity of this uptake process. First, in the presence of leupeptin, the enrichment of ubiquitinylated conjugates was shown to be similar to the enrichment of general markers of autophagy (124). Thus, ubiquitinylated conjugates may not be specifically internalized. Second, no increase in conjugates is apparent in a variety of lysosomal storage diseases (134). Finally, the specific localization of these conjugates to the lysosome and the quantitation of these conjugates depend on immunological techniques. The antiubiquitin antibodies used in these studies react with the polyubiquitin chain better than free ubiquitin, and their reaction with denatured polyubiquitin chains is even stronger. Thus, the apparent enrichment may result from selective exposure of the epitope in the acidic environment of the lysosome. Moreover, Isenman & Dice (72) have shown that some peptides and proteins can survive the lysosomal environment, and ubiquitin is known to be stable to proteolysis. Accumulation of immunoreactivity may result from normal autophagy of ubiquitinylated proteins and from the stability of the polyubiquitin chains in the lysosome. Whatever the interpretation, we know that at least some of the ubiquitinylated proteins in the cell

can be internalized by lysosomes and degraded therein and that assembly of functioning lysosomes requires ubiquitin.

CELLULAR ROLES OF UBIQUITIN-DEPENDENT PROTEIN DEGRADATION

The biological roles of ubiquitin-dependent protein degradation result from the degradation of either short-lived regulatory proteins or abnormal or damaged proteins.

Degradation of Short-Lived Regulatory Proteins

Ubiquitin has been implicated in the degradation of several important regulatory proteins. In some cases, whether the effect is direct (i.e. requiring ubiquitinylation of the target protein) or indirect (e.g. regulating the level of a binding protein or other protease) is unknown. In other cases, the only evidence comes from in vitro studies.

TUMOR SUPPRESSORS AND ONCOGENES The oncogenic papilloma virus E6 gene product stimulates the degradation of the p53 tumor suppressor protein in vivo and in vitro (22). In reticulocyte extracts, E6 from HPV-16 or -18 strains causes the degradation of p53, probably in response to the ubiquitinylation of the p53 protein. The E6 from the low-risk HPV-6 or -11 strains does not bind p53 or cause its degradation. The binding of E6 to the cellular protein, E6-AP, has been directly demonstrated (71), and in vitro degradation of p53 has been reconstituted (22). This pathway apparently uses a novel E2, E2-F1. Recently, the E6-AP protein was purified, sequenced (71), and shown to act as an E3 ligase in the ubiquitinylation of the p53-E6 complex (111). How could such a mechanism have developed? E6 from oncogenic high-risk and low-risk isolates binds to E6-AP, although only the former binds p53 and causes its degradation. This observation suggests that the interaction between E6 and E6-AP may be involved in normal viral infection or reproduction. Oncogenic potential may have been acquired by mutation of E6 in order to allow binding to p53.

The protein products of other nuclear oncogenes, such as N-*myc,* c-*myc,* c-*fos,* and adenovirus E1A, are notable because their protein products are rapidly degraded. Ciechanover et al (19) demonstrated that these proteins are also rapidly degraded in a reticulocyte lysate and that the degradation is dependent on activation of ubiquitin. More recently, Treier et al showed that c-*jun* is degraded by the ubiquitin-dependent system (123). This degradation requires the δ domain, and the corresponding v-*jun,* which lacks this domain, is long lived. Thus, v-*jun* may have evolved to evade the cellular ubiquitin system.

NORMAL TRANSCRIPTION FACTORS: MAT-α2 AND NF-κB The MAT-α2 transcriptional repressor of yeast is a rapidly degraded protein involved in mating type selection (66). The protein contains two domains which, when fused to β-galactosidase, target it for rapid degradation. Immunoprecipitation with antibodies to α2 or epitope-tagged ubiquitin demonstrated that ubiquitin is conjugated to MAT-α2 in vivo. Furthermore, degradation was significantly inhibited by overexpressing inhibitory ubiquitin derivatives (the K48R ubiquitin mutant or the epitope-tagged ubiquitins). Finally, certain null mutants of the UBC conjugating enzymes (E2 carrier proteins) are defective in MAT-α2 degradation (15).

The transcription factor NF-κB plays a key role in the regulation of several important genes involved in the immune response and inflammatory processes. It is formed from a 105-kDa precursor by release of a 50-kDa fragment and by degradation of the C-terminal fragment. The p50 subsequently forms a heterodimer with p65 (RelA). In most cells, this heterodimer is found in the cytoplasm in a ternary complex with IB. Degradation of IκBα in response to a variety of extracellular signals results in the release of NF-κB and its subsequent translocation to the nucleus. Both the processing of p105 to mature p50 and the degradation of IκBα appear to be ubiquitin-dependent processes that require the proteasome (101). The former reaction is unique in the ubiquitin pathway, since a ubiquitinylated protein fragment of significant size (p50) apparently escapes total proteolysis by the proteasome.

CELL-CYCLE PROGRESSION: CYCLINS AND MOS The cyclins are important regulators of cell-cycle progression. They associate with a protein kinase, p34CDC2, to form the heterodimeric complexes. The complex formed with cyclin B, for instance, is an active histone H1 kinase whose presence prevents cells from exiting from mitosis. The degradation rate of cyclins is altered during the cell cycle, with rapid degradation of some cyclins upon exit from mitosis. During interphase, these cyclins gradually accumulate again. Glotzer et al (47) showed that, in *Xenopus* egg extracts, a region of the N-terminus of cyclin that could be fused to other proteins to cause them to be degraded at mitosis was needed to increase the degradation rate. He went on to demonstrate that this degradation was coincident with the conjugation of ubiquitin to cyclin A or to the fusion protein carrying the degradation sequence. Hershko et al (62) then showed that methylated ubiquitin could prevent polyubiquitin chain formation and degradation of cyclins A and B in clam oocyte extracts. Finally, phosphorylation of cyclins may very well occur before ubiquitinylation and degradation (D Finley, unpublished observations).

What triggers the timed degradation of the cyclins remains unknown. The exit from M phase upon fertilization of vertebrate eggs requires degradation of cyclins and is prevented by the presence of p39mos (88). Upon fertilization,

intracellular calcium increases to micromolar levels; cyclins are degraded; and mitosis occurs. This same calcium transient, when applied to *Xenopus* oocyte lysates, allows cyclin degradation to take place. This degradation can be inhibited by adding a calcium-calmodulin binding peptide and restored by adding calmodulin. These results implicate phosphorylation as a regulatory event, but there is little direct evidence to support this hypothesis. Degradation of p39mos has been shown to involve ubiquitinylation (99) and to be catalyzed by the proteasome (98). Phosphorylation of ser-3 prevents this ubiquitinylation and degradation. Thus, exit from mitosis may be regulated by phosphorylation of cyclin, which enhances its degradation and allows exit, or by phosphorylation of p39mos, which prevents its degradation and subsequently that of cyclin.

CONFORMATIONALCHANGES:PHYTOCHROME A major photoreceptor in plants is phytochrome. It consists of a 124-kDa polypeptide with a linear tetrapyrrole chromophore and is involved in the control of red light–mediated morphogenesis. A brief pulse of light converts the red light–absorbing form (Pr) to a far–red light–absorbing form (Pfr), which then aggregates. The half-life of Pr is more than 100 h, whereas Pfr is degraded with a half-life of approximately 2 h. The enhanced degradation correlates with the conjugation of ubiquitin (73). The signal that causes increased ubiquitinylation and degradation in this system appears to be a conformational change associated with a light-induced isomerization of the chromophore. This altered conformation results in increased sensitivity to trypsin, aggregation, and ubiquitinylation of both the soluble and the aggregated protein. This mechanism may recognize conformational changes resulting from ligand binding, from translocation to membrane surfaces, or from binding of allosteric regulators and may degrade such proteins in response to these cellular signals.

Degradation of Abnormal Proteins

Another role of the ubiquitin-dependent system is that of "garbage collector" for damaged protein in the cell. This stress response may work in concert with heat-shock proteins and/or remove those proteins that cannot be repaired by the system.

AMINO ACID ANALOGUES A number of early studies on intracellular proteolysis demonstrated that the incorporation of amino acid analogues such as canavanine led to the synthesis of short-lived abnormal proteins (60). Degradation of these proteins in hepatoma cells correlated with the appearance of ubiquitin conjugates of the analogue-containing proteins (61). Specific mutants have indicated that a significant portion of the degradation of short-lived and analogue-containing proteins requires functional ubiquitin-activating enzyme E1 (20), E2 carrier proteins (112), E3 ligase (9), and the proteasome (58).

OXIDIZED, DAMAGED, OR DENATURED PROTEINS The degradation rate of normal proteins has been correlated with size, stability, and susceptibility to proteases. This association led to the suggestion that denaturation, especially that caused by damage, may be an important signal for proteolysis. Unless otherwise referenced, the following examples have been discussed in recent reviews (21, 106, 126, 130). Denaturation of hémoglobin with phenylhydrazine results in increased ubiquitinylation of the protein, and the levels of ubiquitinylation correlate with the degradation rate. Aggregation of phytochrome Pfr or alkylated BSA also apparently triggers ubiquitinylation. Reduction of a single disulfide bond is sufficient to cause a slight conformational change as well as the ubiquitinylation and subsequent degradation of lysozyme. Oxidation of methionines in ribonuclease and lysozyme greatly increases the rate of ubiquitinylation and degradation in the reticulocyte system, probably by increasing binding to the protein substrate binding site of E3 ubiquitin protein ligase. Oxidation of hexokinase and hemoglobin (46) causes rapid degradation by the ubiquitin-dependent system. Finally, three different cytochrome P450 isozymes have been reported to be rapidly degraded by the ubiquitin-dependent system after mechanism-based inactivation (23, 120).

These data point to a mechanism that distinguishes normal from damaged proteins. The proposed proofreading mechanism described above may be involved. In this model, proteins are semirandomly ubiquitinylated. Protein damage (possibly sensed by exposure of hydrophobic regions) would cause a conformational change in the first ubiquitin attached and would thus signal polyubiquitinylation. If no damage were sensed, ubiquitin would remain in the native conformation and be removed by one of the ubiquitin-specific proteases.

OTHER POSSIBLE ROLES OF UBIQUITINYLATION

The processes discussed above all involve complete proteolytic degradation of target proteins. However, several cases of partial degradation or limited ubiquitinylation have been observed. Below we discuss the possible role of these more limited pathways.

Antigen Presentation

Class I antigen presentation is responsible for self and nonself immune responses. Among the best antigens are viral-encoded nuclear proteins. Considerable evidence indicates that these proteins are degraded to peptides. These peptides are then taken up into the endoplasmic reticulum by peptide transporters, where they assemble with newly synthesized class I MHC molecules. After efflux of the antigen-MHC complex to the surface of the cell, these cells are destroyed by cytotoxic T lymphocytes. Normal cellular proteins probably

also follow this pathway, but these peptides are not antigenic because self-reactive T lymphocytes were removed early in development (32).

This proteolysis has long been attributed to the ubiquitin-dependent proteolysis system (48). The first experimental evidence supporting this hypothesis came from the observation that adding a signal for ubiquitinylation to the N-terminus of nucleoprotein enhanced the efficiency of its presentation (122). Moreover, two MHC-encoded genes, RING10 (LMP2) and RING12 (LMP7), are apparently members of the prosome β gene family (32) and, like antigen presentation, are induced by γ-interferon (1, 10, 40, 107). The yeast homologue of RING10 (PRE2) encodes a subunit with chymotryptic-like activity, whereas the yeast homologue of RING12 (PRE3) encodes a peptidylglutamyl-peptide hydrolyzing activity. Both homologues are required for degradation of ubiquitinylated proteins (35, 57). Protease inhibitors, which affect cathepsins, calpains, and proteasomes, also prevent efficient antigen presentation (109). Finally, the substrate specificity of proteasome and that of antigen presentation are somewhat correlated (10, 31, 42).

Thus, a reasonable model invokes the proteasome as the protease that degrades cytoplasmic and nuclear proteins to generate limited peptides. These peptides are then transported to the endoplasmic reticulum (ER) by peptide transporters. Evidence for a ubiquitinylation step comes from the finding that antigen presentation is impaired in ts-85 cells at the nonpermissive temperature (92). This cell line is defective in ubiquitin activation and conjugation, but because the mutation is pleiotrophic, the evidence for a necessary and sufficient ubiquitinylation step is not yet compelling.

Receptor Structure and Function

Previous reviews (21, 60, 65, 74, 106) have discussed the ubiquitinylation of cell-surface receptors. Those known to be ubiquitinylated include the lymphocyte homing receptor (125), the platelet-derived growth factor (PDGF) receptor (95), the growth hormone receptor (116), the T-cell antigen receptor (13), the high-affinity immunoglobulin E (IgE) receptor (102), the human tumor necrosis factor receptor (87), the c-*kit* receptor (94), and a 67-kDa adhesion receptor of the marine sponge (97). The lymphocyte homing receptor is ubiquitinylated early in biosynthesis, perhaps cotranslationally (125). The T-cell receptor, the PDGF receptor, the IgE receptors, and the c-*kit* receptor are polyubiquitinylated in response to binding of their respective physiological ligands. The PDGF receptor and the c-*kit* receptors are apparently degraded after polyubiquitinylation. This process appears to require endocytosis of the receptor. In an analogous yeast system, uracil permease is endocytosed in response to stress, and a cyclin-like degradation sequence is required for its degradation (43). In contrast, the IgE receptor is reversibly modified, with no evidence for its involvement in degradation.

From this small sampling, one function of ubiquitinylation appears to be the downregulation of receptors. Indeed, at least one E2 (UbC6) is known to be localized in the ER and to affect membrane protein transport (115). Ubiquitinylation may also be involved in extracellular events. For example, monoclonal antibodies to ubiquitin decorate the surface of cells; a ubiquitin binding site has been identified on hemopoietic progenitor cells (104); and lymphocyte homing can be blocked by ubiquitin or antibodies to ubiquitin. Finally, the reversible ubiquitinylation on the cytoplasmic face of a receptor may indicate participation in signal transduction events (102).

Chromosome Structure and Function

The first ubiquitinylated protein discovered was A-24, a ubiquitinylated histone H2a. Approximately 10% of the H2a molecules are ubiquitinylated in interphase, and ubiquitin is removed from A-24 during mitosis (11). This ubiquitinylation/deubiquitinylation is rapid and reversible. The modification is not apparent in yeast and is not required (117). Several groups have suggested that histones of transcriptionally active chromatin are preferentially ubiquitinylated, and Davie & Murphy (27) recently showed that this process requires active transcription. Because histone proteins are thought to be stable, this reversible modification likely has some structural implications for transcriptional activity or chromatin condensation. Indeed, at nonpermissive temperature, E1 conditional mutants are cell-cycle arrested and fail to condense their chromatin (21, 60).

Ubiquitinylation also appears to play a role in DNA repair. The yeast E2 carrier protein, UBC2, is encoded by the RAD6 locus. Mutants in this locus are defective in DNA repair. Since repair of DNA lesions probably requires extensive alterations in chromatin structure, these two functions are difficult to separate. Similarly, the CDC34 mutation (another E2 carrier protein) may affect chromatin structure, as it has been argued that most structural modifications of chromosomes will be involved in cell-cycle control (11).

Vectoral Transport

A considerably more speculative example of putative ubiquitin functions is that of vectoral protein transport. Such transport would be necessary to generate immunoreactive ubiquitin on the surface of cells, to target ubiquitinylated proteins to the lysosome, or to ubiquitinylate proteins in various organelles. Zhuang et al have reported that mitochondrial import of monoamine oxidase is inhibited by antibodies to ubiquitin and that this inhibition can be relieved by addition of ubiquitin (136). Interestingly, two yeast E2 carrier proteins have been implicated in similar processes (74). UBC6 is reported to be an integral membrane protein of the ER involved in secretion (115), and UBC10 (PAS2) has been implicated in peroxisome biogenesis (128). STE-6, the peptide trans-

porter necessary for the secretion of yeast MAT-α, is normally found associated with the golgi in yeast. In secretory mutants, it accumulates in the plasma membrane as a ubiquitinylated species (79) and is rapidly degraded via endocytosis and vacuolar proteolysis. Finally, a significant variety of ubiquitinylated proteins has been reported in postsynaptic vesicles (14) as well as in other cellular organelles (90), suggesting an extensive role for ubiquitinylation in membrane structure and function. Whether that role is attributable to proteolysis, or to other signaling pathways, remains unknown.

It is easy to see how the machinery of this proteolysis system could accomplish vectoral transport. A ubiquitin binding protein could deliver the conjugate to a transporter (perhaps a pore resembling a modified 20S prosome). The action of a ubiquitin carboxyl-terminal hydrolase could remove the ubiquitin signal coincident with or after transversal of the membrane. We have noted, but not identified, membrane-bound forms of UCH immunoreactivity (131).

Viral Defense Mechanisms

Viruses have clearly usurped the ubiquitin-dependent system in ill-defined ways to assist in their survival and replication. Degradation of p53 by the ubiquitin system is triggered by papilloma virus infection (see above). Herpes infection induces transcription of proubiquitin by the *trans*-activating factor ICP4 (83). Tobacco mosaic virus (TMV) particles contain on average one ubiquitin covalently attached to the coat protein (34). Several viral genes encoding ubiquitin-related proteins (reviewed in 75) have been described, although their functions are less clear. Two RNA viruses have a ubiquitin-like gene that encodes a protein fusion with viral protein sequences (53, 91). Whether these sequences are proteolytically processed is unknown, but cytopathogenicity appears to be correlated with the presence of the ubiquitin-like sequence (91). In bacculovirus, a DNA virus, a single copy of ubiquitin is encoded with tyrosine as a C-terminal extension. This viral protein is processed to an active ubiquitin, although conjugates of this viral ubiquitin are formed and degraded more slowly than those with eukaryotic ubiquitin (A Haas & L Guardino, privileged communication).

Another sequence related to viral infection is the human interferon–induced 15-kDa protein, which appears to have resulted from gene duplication. This protein consists of an evolutionarily diverged N-terminal copy of ubiquitin, a more highly conserved C-terminal copy of ubiquitin, and a nine–amino acid extension. The nonapeptide is proteolytically cleaved to produce the 15-kDa protein, which has been shown to be conjugated to cellular proteins and to be present at low levels in normal cells (86). Thus, under the stress of a viral infection, these ubiquitin-like sequences are expressed and may be involved in viral pathogenicity or in cellular response to interferons (see below). The interferon-induced 15-kDa ubiquitin homologue can be conjugated to proteins,

although the precise consequences of this association are unknown (86). Finally, an E2 carrier protein homologous to the RAD6 and CDC34 gene products is encoded by an African swine fever virus (64).

PERSPECTIVES

These viral "tricks" point out the potential for manipulating important cellular functions by modifying ubiquitin metabolism. Perhaps one of the most obvious conclusions that can be drawn from the above discussion is that this proteolytic pathway is exquisitely regulated. The degradation of specific proteins depends variously on the presence or absence of a signaling sequence, the phosphorylation state of that sequence, the conformational changes induced by ligand binding, and/or a variety of damage events. Both host- and pathogen-encoded gene products are used in some circumstances, leading to a complex interplay. For instance, the interactions of this degradation system with the cellular stress response are unclear at best, but a hypothesis can be formulated. Stress proteins are thought to be involved in repair of damage, perhaps by acting as catalysts for refolding proteins. Evidence indicates that the constitutive homologues often are important for proper folding or localization of proteins. If refolding can be accomplished by the stress proteins, the cell survives. The ubiquitin system may only be invoked when that system fails and may even involve the complex with heat-shock proteins (HSPs) as signals for ubiquitinylation. If the ubiquitin system fails, the cell dies. The presence of ubiquitin in lysosomes and in a variety of neural pathogenic lesions may be the "scar" left by this unsuccessful attempt to repair the damage.

That such redundancy occurs in the enzymes of ubiquitinylation and deubiquitinylation is also striking. Database searches suggest that at least a dozen of each of these types of enzymes are present. Knockouts or mutations in several of the E2 conjugating enzymes have very limited defined phenotypes, which suggests that each E2 may have a limited substrate specificity and that several parallel pathways of ubiquitinylation (and subsequent fates) may exist. Each pathway may also utilize one or more members of the UBP family of deconjugating enzymes. This futile cycle is reminiscent of the cellular regulation afforded by paired protein kinases and phosphatases.

Finally, although the genetics of the system in yeast has been tremendously important, it cannot completely elucidate the complex developmental biology of vertebrates or the repertoire of cellular responses of a multicellular organism. The analysis of these genes must be applied to more complex organisms. A start has been made in *Drosophila,* and progress in the field of transgenic mammals will likely be forthcoming. The incredible redundancy of the system makes a number of interesting and informative mutants likely.

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