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The ubiquitin–proteasome pathway: on protein death and cell life

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Introduction

The discovery of the ubiquitin pathway and its many substrates and functions has revolutionized our concept of intracellular protein degradation. From an unregulated, non-specific terminal scavenger process, it has become clear that proteolysis of cellular proteins is a highly complex, temporally controlled and tightly regulated process which plays important roles in a broad array of basic cellular processes. It is carried out by a complex cascade of enzymes and displays a high degree of specificity towards its numerous substrates. Among these are cell cycle and growth regulators, components of signal transduction pathways, enzymes of house keeping and cell-specific metabolic pathways, and mutated or post-translationally damaged proteins. The system is also involved in processing major histocompatibility complex (MHC) class I antigens. For many years it has been thought that activity of the system is limited to the cytosol and probably to the nucleus. However, recent experimental evidence has demonstrated that membrane-anchored and even secretory pathway-compartmentalized proteins are also targeted by the system. These proteins must be first translocated in a retrograde manner into the cytosol, as components of the pathway have not been identified in the endoplasmic reticulum (ER) lumen. With the multiple cellular targets, it is not surprising that the system is involved in the regulation of many basic cellular processes such as cell cycle and division, differentiation and development, the response to stress and extracellular modulators, morphogenesis of neuronal networks, modulation of cell surface receptors, ion channels and the secretory pathway, DNA repair, regulation of the immune and inflammatory responses, biogenesis of organelles and apoptosis. One would also predict that aberrations in such a complex system may be implicated in the pathogenesis of many diseases, both inherited and acquired. Recent evidence shows that this is indeed the case.

Degradation of a protein by the ubiquitin system involves two distinct and successive steps: (i) covalent attachment of multiple ubiquitin molecules to the target protein (Figure 1A); and (ii) degradation of the tagged protein by the 26S proteasome (Figure 1B) or, in certain cases, by the lysosomes/vacuole. Conjugation of ubiquitin to the substrate proceeds via a three-step mechanism. Initially, ubiquitin is activated in its C-terminal Gly by the ubiquitin-activating enzyme, E1. Following activation, one of several E2 enzymes (ubiquitin-carrier proteins or ubiquitin-conjugating enzymes, UBCs) transfers ubiquitin from E1 to a member of the ubiquitin-protein ligase family, E3, to which the substrate protein is specifically bound. This enzyme catalyzes the last step in the conjugation process, covalent attachment of ubiquitin to the substrate. The first moiety is transferred to an ε-NH_2 group of a Lys residue of the protein substrate to generate an isopeptide bond. The first moiety can be also conjugated in a linear manner to the N-terminal residue of the substrate (Breitschopf *et al.*, 1998). In successive reactions, a polyubiquitin chain is synthesized by transfer of additional ubiquitin moieties to Lys48 of the previously conjugated molecule. The chain serves, most probably, as a recognition marker for the protease. The structure of the system appears to be hierarchical (Figure 2): a single E1 activates ubiquitin required for all modifications. It can transfer ubiquitin to several species of E2 enzymes, and each E2 acts with either one or several E3s. Only a few E3s have been identified so far, but it appears that these enzymes belong to a large and rapidly growing family of proteins. A major, as yet unresolved problem involves the mechanisms that underlie the high specificity and selectivity of the system. Why are certain proteins extremely stable while others are exceedingly short-lived? Why are certain proteins degraded at a particular time point in the cell cycle or only following specific extracellular stimuli, while they are stable under all other physiological conditions? It appears that specificity is determined by two distinct groups of proteins. Within the ubiquitin system, substrates are recognized by the different E3s. Some proteins are recognized via primary signals and bind directly to E3s. However, many proteins must undergo post-translational modification such as phosphorylation, or associate with ancillary proteins such as molecular chaperones prior to recognition by the appropriate ligase (for modes of substrate recognition, see Figure 3). Thus, the modifying enzymes and ancillary proteins also play an important role in the recognition process. As for the E3s, except for a few cases, it is not likely that each substrate is targeted by a single ligase; rather, it is conceivable that a single E3 recognizes a subset of similar, but clearly not identical, structural motifs.

The exponential increase of information on the ubiquitin system has made it impossible to describe all the important advances in the field in a single review, however compre-

This review is dedicated to the memory of Thomas Kreis, a wonderful colleague and a superb scientist, who was killed in the crash of Swissair Flight 111 on September 2, 1998.

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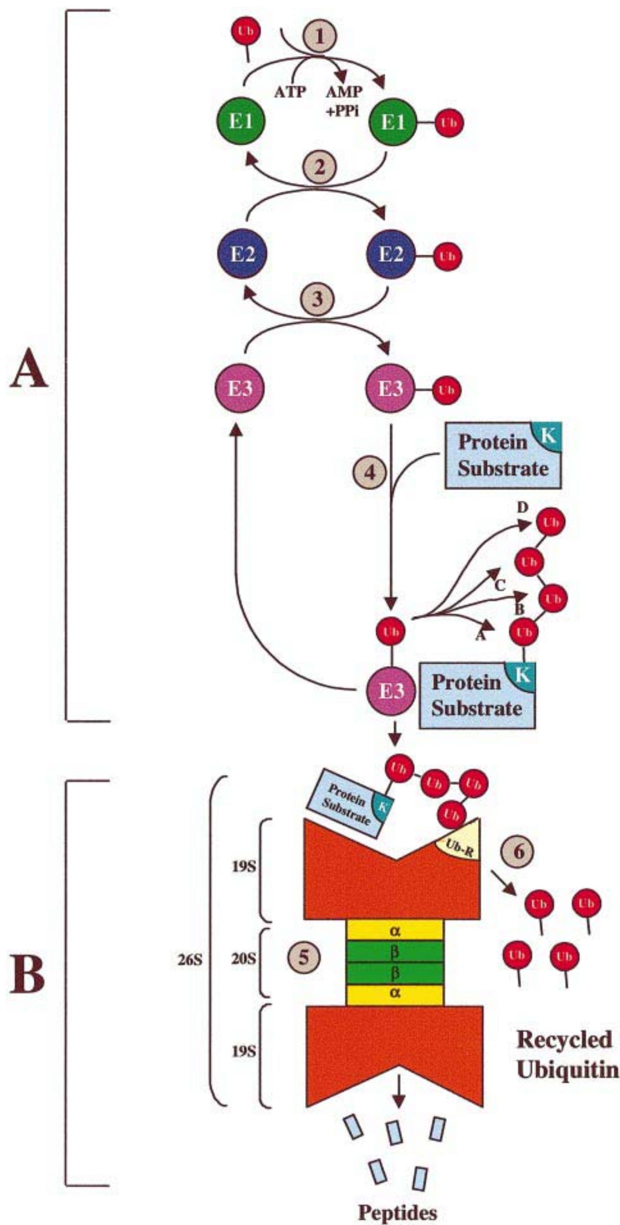

Fig. 1. The ubiquitin–proteasome pathway. (**A**) conjugation of ubiquitin to the target molecule. (**B**) Degradation of the tagged substrate by the 26S proteasome. (1) Activation of ubiquitin by E1. (2) Transfer of activated ubiquitin from E1 to a member of the E2 family. (3) Transfer of activated ubiquitin from E2 to a substrate-specific E3. (4) Formation of a substrate–E3 complex and biosynthesis of a substrate-anchored polyubiquitin chain. (5) Binding of the polyubiquitinated substrate to the ubiquitin receptor subunit in the 19S complex of the 26S proteasome and degradation of the substrate to short peptides by the 20S complex. (6) Recycling of ubiquitin via the action of isopeptidases.

hensive. Many recent review articles and monographs have described different aspects of the pathway (see, for example, Coux *et al.*, 1996; Hochstrasser, 1996; Baumeister *et al.*, 1998; Hershko and Ciechanover, 1998; Peters *et al.*, 1998). Here, I shall summarize for the novice reader the enzymes and mechanisms involved in ubiquitin-mediated proteolysis and describe some recent advances in the pathophysiology of the system.

The ubiquitin system cascade

Ubiquitin-conjugating machinery

E1. This enzyme generates a high-energy thiolester intermediate with ubiquitin that involves an internal Cys residue.

E2s, UBCs. The activated ubiquitin is transferred from E1 to a Cys residue of an E2 enzyme, thereby generating yet another thiolester intermediate. The genome of *Saccharomyces cerevisiae* encodes for 13 E2s and E2-like proteins, and many more have been described in mammals. Some E2s are involved in specific cellular processes while the role of others is still obscure. However, they all act via their function as UBCs: all are inactivated by mutation of the active Cys residue. The yeast UBC2/RAD6 is involved in degradation of 'N-end rule' substrates and also in DNA repair. The mechanism that underlies this activity is still obscure. UBC3/CDC34 is required for $G_1 \rightarrow S$ transition, probably via degradation of certain cell-cycle regulators, while UBC4 and UBC5 are involved in the degradation of many short-lived, normal and abnormal proteins. E2-C acts along with the cyclosome/anaphase promoting complex (APC) in the degradation of some cell-cycle regulators. *Drosophila* UBCD1 is involved, probably via degradation of some telomere-associated proteins, in proper detachment of telomeres during mitosis and meiosis. The *Drosophila bendless* gene encodes an E2 enzyme required for the formation of synaptic networks. HRB6B, one of the two mouse homologs of the yeast UBC2/RAD6, is involved in degradation of histones occurring during spermatogenesis (see below). Disruption of *UBCM4*, a mouse homolog of yeast UBC4/UBC5, causes embryonic lethality which is probably due to impairment in the development of the placenta (reviewed in Hershko and Ciechanover, 1998). Other E2s are membrane-associated and may be involved in degradation of abnormal or virus-targeted ER proteins (see below). One membrane-associated E2 contains a baculovirus inhibitor of apoptosis repeat, suggesting that these enzymes are structurally and functionally more diverse, and play a role in more than one process (Hauser *et al.*, 1998).

Due to the specific effects of certain E2s on defined processes, it has been proposed that they can interact directly with the substrate protein. While such interactions have been described using protein–protein interaction screening methods, their physiological significance is not clear. Most probably, the specific functions of E2s are due to their association with distinct E3s (reviewed in Hershko and Ciechanover, 1998).

E3s. An E3 enzyme is defined as a protein that binds the target substrates, either directly or indirectly, via ancillary proteins, and catalyzes transfer of ubiquitin from a thiolester intermediate on E2 or E3 to an amide linkage with the substrate or with a polyubiquitin chain already anchored to it. Since the target proteins bind to the ligases prior to conjugation, E3s are key players in determining the high specificity of the system. Despite their importance, the number of known E3s is few and the information concerning their mode of action is rarer. Lack of sequence homology among different E3s and the frequent association of these enzymes with multisubunit complexes in

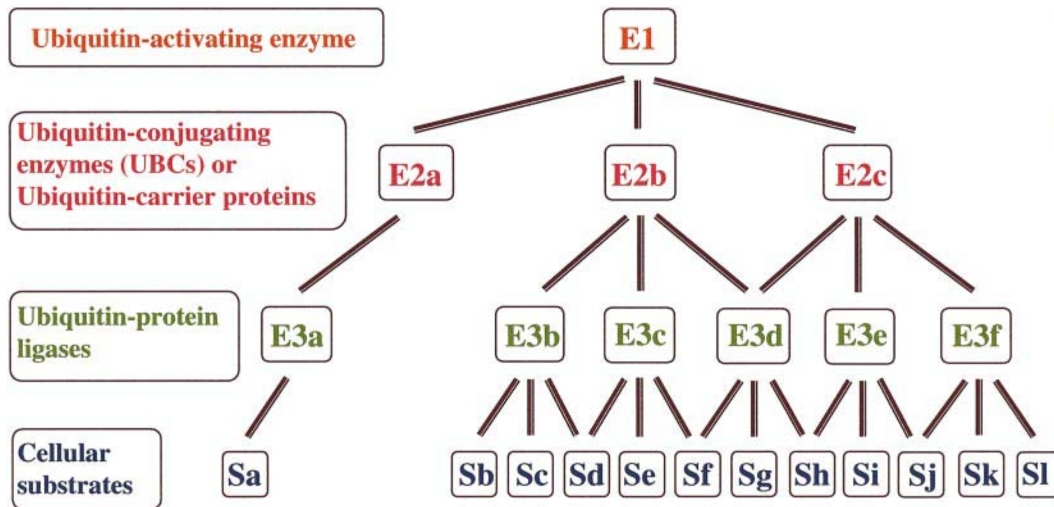

Fig. 2. Hierarchical structure of the ubiquitin-conjugating machinery. A single E1 catalyzes activation of ubiquitin and transfers it to several E2 enzymes. In most cases, an E2 transfers ubiquitin to several E3s, while in a few cases the E2 is E3-specific. E3s can be substrate-specific or can recognize several substrates via similar, but not identical motifs. Certain substrates can be targeted by several E3s, probably via distinct recognition motifs.

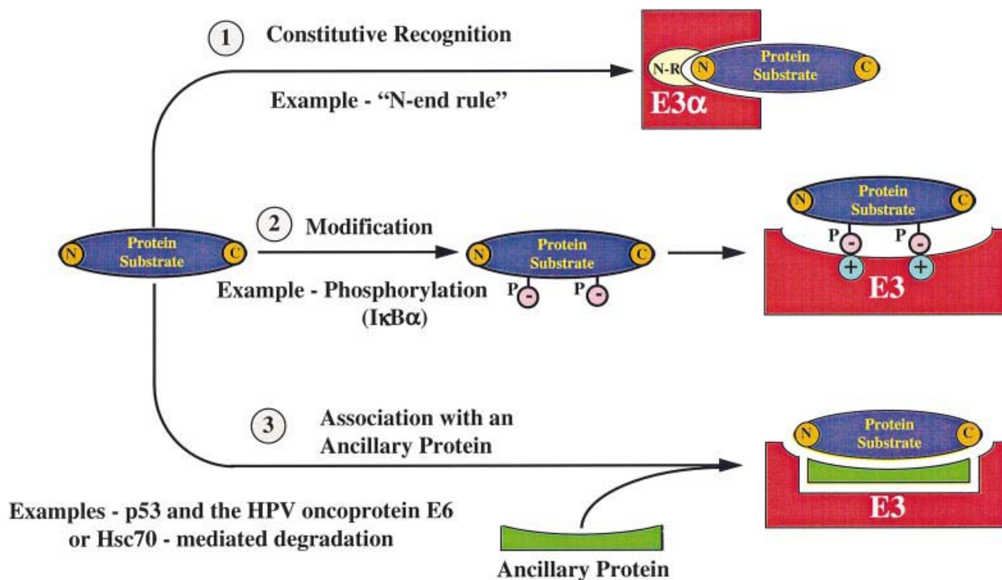

Fig. 3. Modes of recognition of protein substrates by different E3s. An E3 can recognize a substrate constitutively via a primary motif such as the N-terminal residue (N-end rule). Many proteins are recognized following post-translational modification (e.g. phosphorylation) or association with an ancillary protein (e.g. Hsc or HPV-E6). N-R, N-terminal receptor.

which the identity of the ligase subunit is not known, render their study difficult.

The four families of E3 enzymes that have been described so far are as follows. (i) The main N-end rule E3, E3α, and its yeast counterpart UBR1, contain two distinct sites that recognize either basic (Type I) or bulky-hydrophobic (Type II) N-terminal residues of their substrates. However, they also recognize non-N-end rule substrates such as N-α-acetylated proteins that bind via a yet uncharacterized ‘body’ site. E3β is a related enzyme that binds proteins with small uncharged N-termini. Although the N-end rule recognition mechanism is highly conserved, its cellular substrates and physiological roles are still obscure (reviewed in Varshavsky, 1996). (ii) A second group is the HECT (homologous to E6-AP C-terminus) domain family. One member of the family, E6-AP (E6-associated protein) is required, along with the human papillomavirus (HPV) E6 oncoprotein, for the ubiquitination and degradation of p53 (Scheffner *et al.*, 1993). The enzyme recognizes p53 in a *trans* manner following formation of a ternary complex with E6 that recognizes both the ligase and the tumor suppressor. A large family of proteins that contain a HECT-domain has been identified in many eukaryotes (Huibregtse *et al.*, 1995). The C-terminal domain that contains the ubiquitin-binding Cys residue is highly conserved, whereas the N-terminal region of the various HECT proteins is variable and is probably involved in specific substrate recognition. Members of the family are involved in the targeting of specific proteins. For example, yeast RSP5 conjugates the large subunit of RNA polymerase II (Huibregtse *et al.*, 1997) and also the FUR4 uracil permease which is targeted to the vacuole following ubiquitination (Galan *et al.*, 1996). NEDD4 targets the kidney epithelial sodium chan-

nel (ENaC; Staub *et al.*, 1997; see below). (iii) A third type of ligase is the ~1500 kDa cyclosome (Sudakin *et al.*, 1995) or anaphase promoting complex (APC; King *et al.*, 1995). This complex has a ubiquitin ligase activity specific for cell-cycle regulators, such as mitotic cyclins, certain anaphase inhibitors and spindle-associated proteins, that contain a nine amino acid motif designated the 'destruction box' (see below) and are degraded during mitosis. The complex is inactive during interphase. At the end of mitosis it is activated by phosphorylation mediated by the cyclin-B/cyclin-dependent kinase (CDK)1 complex MPF (M-phase promoting factor; Lahav-Baratz *et al.*, 1995). The *Xenopus* complex has eight subunits, three of which are homologous to *S.cerevisiae* CDC16, CDC23 and CDC27, which are required for exit from mitosis and for the degradation of B-type cyclins. A fourth subunit is homologous to *Aspergillus* BimE which is essential for completion of mitosis. The subunit of the cyclosome involved in its ubiquitin ligase function has not been identified. (iv) A different type of multi-subunit ubiquitin ligase is involved in the degradation of certain other cell-cycle regulators, such as the SIC1 CDK inhibitor or certain G_1 cyclins. Here, phosphorylation of the substrate converts it to a form susceptible to the action of the ligase complex. Several such complexes, designated Skp1-cullin-F-box protein ligase complexes (SCFs), have been described that share some common subunits, but also contain distinct subunits specific for certain substrates. Thus, the degradation of the CDK inhibitor SIC1, a process essential for $G_1 \rightarrow S$ transition in yeast, requires its phosphorylation by a G_1 cyclin-activated kinase as well as the products of *CDC34*, *CDC53*, *CDC4* and *SKP1* genes. CDC34 is an E2, but the role of the other proteins is not known. CDC34, CDC53 and CDC4 generate a complex that is responsible for ubiquitination of phosphorylated SIC1 (Feldman *et al.*, 1997; Skowyra *et al.*, 1997). Ubiquitination and degradation of the yeast G_1 cyclin CLN2, also requires its phosphorylation and the action of CDC34, CDC53, GRR1 and SKP1, but not of CDC4. Both CDC4 and GRR1 contain a motif called the F-box that is present in a variety of proteins that bind to SKP1. It was proposed that SKP1 is a component of SCF complexes that binds to specific 'adaptor' proteins such as CDC4 and GRR1, which in turn bind to specific protein substrates such as phosphorylated SIC1 and CLN2 (reviewed in Hershko and Ciechanover, 1998). Components of the SCF complexes are highly conserved during evolution and have been identified in organisms ranging from *Caenorhabditis elegans* to human. They are designated cullins, and their existence suggests that similar complexes may be involved in the degradation of a variety of regulators in higher organisms.

Structural motifs that target proteins for ubiquitination

The numerous substrates of the pathway are recognized by the different ligases via specific motifs. These can be either primary, or secondary, post-translational modifications. Primary motifs do not necessarily lead to constitutive degradation of the proteins that contain them. They can be hidden and exposed only following misfolding or dissociation of subunits. For example, masking of a degradation signal by heterodimerization blocks the proteolysis of the MATα2·MATa1 heterodimeric yeast transcription factor (Johnson *et al.*, 1998). Binding to its specific DNA promoter blocks the degradation of MyoD (Abu Hatoum *et al.*, 1998). Certain substrates will not be recognized by their ligases unless they associate with an ancillary protein or molecular chaperone that act as *trans* recognition elements.

The best studied primary signal is the N-terminal residue (N-end rule; Varshavsky, 1996). Association with ancillary proteins such as viral oncoproteins or molecular chaperones has been shown to accelerate the degradation of certain substrates (reviewed in Hershko and Ciechanover, 1998). As for secondary motifs, recent evidence indicates that many proteins are targeted by phosphorylation. The yeast G_1 cyclin CLN3 (Yaglom *et al.*, 1995) and the GCN4 transcriptional activator (Kornitzer *et al.*, 1994) are degraded following phosphorylation at a PEST sequence. The mammalian G_1 cyclin D1 is targeted for degradation following phosphorylation on a Thr residue that does not reside within a PEST [Pro(P), Glu(E), Ser(S), Thr(T)] sequence (Diehl *et al.*, 1997). Phosphorylation of Ser32 and Ser36 targets IκBα (Chen *et al.*, 1995). Here, the phosphorylation site constitutes the ligase-binding site (Yaron *et al.*, 1997). Degradation of β-catenin is also mediated by phosphorylation at Ser37 which resides in a region similar to the targeting domain of IκBα (Rubinfeld *et al.*, 1997; see below). Phosphorylation at Ser3 of c-Mos (Nishizawa *et al.*, 1992) or multiple phosphorylations of c-Jun (Musti *et al.*, 1997) suppress their ubiquitination and degradation. Ligand binding to Ste2p, the G protein-coupled membrane receptor of the α factor, leads to phosphorylation of Ser residues that reside on a well defined internalization signal, SINNDAKSS. Phosphorylation signals ubiquitination which is required for internalization of the ligand–receptor complex (Hicke *et al.*, 1998).

An important degradation signal, the 'destruction box', was discovered in mitotic cyclins and certain other cell-cycle regulators. It is a nine-amino-acid motif, usually located ~40–50 amino acid residues from the N-terminus. It has the following general structure: $R_1(A/T)_2(A)_3 L_4(G)_5X_6(I/V)_7(G/T)_8(N)_9$. Amino acid residues shown in brackets occur in most known destruction sequences. R_1 and L_4 are indispensable. Cyclosome-mediated ubiquitination of destruction box-containing proteins is an example of a limited set of proteins that perform related functions, share a common targeting signal and are recognized by a common E3. The mechanistic role of the destruction box is not known. It does not serve as a phosphorylation or ubiquitination site but may serve as a 'docking' domain for the E3 subunit of the cyclosome.

In most cases, the Lys residues that serve as ubiquitination sites are not specific or part of the recognition motif. In the case of IκBα, however, Lys21 and 22 are indispensable (Scherer *et al.*, 1995), although they are not part of the E3-binding site (Yaron *et al.*, 1997).

Conjugation of ubiquitin to cell-surface membrane proteins, such as the growth hormone receptor (Strous *et al.*, 1996), leads to their targeting to the lysosome. The mechanism(s) and signals that underlie this unique trafficking are not known. In the case of certain membrane proteins, formation of the polyubiquitin chain can proceed via Lys63 (Galan and Haguenauer-Tsapis, 1997), while in

others monoubiquitination appears to be sufficient for targeting (Terrel *et al.*, 1998).

Ubiquitin conjugates degrading enzymes

The 20S and 26S proteasomes. The 26S proteasome is composed of the 20S core catalytic complex flanked on both sides by the 19S regulatory complexes. With one known exception (ornithine decarboxylase which is proteolyzed following association with its inhibitor antizyme but without prior ubiquitination) the 26S complex recognizes specifically ubiquitin-tagged proteins.

An important advance in studies of the 26S complex has been the resolution of the crystal structure of the yeast 20S proteasome at 2.4 Å (Groll *et al.*, 1997). The complex is arranged as a stack of four rings, two α and two β, organized in the general structure of $\alpha\beta\beta\alpha$. Both α and β rings are composed of seven distinct subunits. Thus, the general structure of the complex is $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$. The three catalytic sites: the trypsin-, chymotrypsin- and post-glutamyl peptidyl hydrolytic-like sites, reside on some of the β subunits, and are generated topologically by obliquely adjacent pairs of identical β subunits residing in different β rings. The crystal structure has also shown that the catalytically inactive α chains play an essential role in stabilizing the two-ring structure of the β chains. They also play a role in the binding of the 19S 'cap' regulatory complexes.

An important, as yet unresolved, problem involves the entry of substrates and exit of proteolysis products from the proteasome. In the *Thermoplasma* proteasome, there are two entry pores at the ends of the cylinder. These pores do not exist in the yeast 20S proteasome: the N-terminal domains of the α subunits protrude towards each other and fill the space. Entry from the ends may be possible only following substantial ATP-dependent rearrangement that may occur following association with the 19S complex. The yeast complex displays narrow side orifices at the interface between the α and β rings. These openings lead directly to the active sites. They can potentially rearrange to generate entry apertures for unfolded/extended substrates.

Substrate recognition by the 26S proteasome is probably mediated by the interaction of specific subunits of the 19S complex with the polyubiquitin chain. Polyubiquitin-binding subunits have been described in human (S5a), yeast (RPN10; MCB1) and plants (MBP1). Surprisingly, Δ*mcb1* yeast mutant does not display any growth defect and degrades normally the vast majority of ubiquitinated proteins. The mutant also displays a slight sensitivity to stress (van Nocker *et al.*, 1996). It is possible that ubiquitinated proteins are recognized by an additional, at present undefined proteasomal subunit.

An additional complex that associates with the 20S proteasome is PA28 (REG or 11S; Song *et al.*, 1997). Unlike assembly of the 19S–20S–19S, complex formation with PA28 is ATP-independent, the PA28–20S–PA28 complex digests only peptides but not ubiquitin-conjugated intact proteins. The activator is a ring-shaped hexamer composed of alternating α and β subunits. Both subunits are induced by IFN-γ, suggesting a role for the particle in antigen processing. Indeed, overexpression of PA28α in cell lines that also express viral protein antigens results in an enhanced presentation of peptides derived from these proteins (Groettrup *et al.*, 1996). Since the PA28–20S–PA28 proteasome cannot digest intact proteins, it must act downstream to the 26S proteasome. It can act in trimming large peptides that were generated by the 26S complex to the precise epitopes recognized by the class I MHC complex and T-cell receptors. The existence of a single, asymmetrical 19S–20S–PA28 proteasome has been reported (Hendil *et al.*, 1998). Such a complex has the potential to carry out, in a consecutive manner, the two-step proteolytic processes, initial proteolysis to large peptides and final trimming to the antigenic peptides.

An important development involves the discovery of proteasome inhibitors which have become powerful research tools in probing the structure and function of the proteasome and the ubiquitin pathway. The first inhibitors were derivatives of the calpain inhibitors I [N-Acetyl-Leu-Leu-Norleucinal (ALLN)] and II [N-Acetyl-Leu-Leu-Methioninal (ALLM)]. These inhibitors block degradation of the bulk of cellular proteins, short- and long-lived alike (Rock *et al.*, 1994), suggesting that the vast majority of cellular proteins are targeted by the system. While these inhibitors are quite specific, they also inhibit calpains. In contrast, the *Streptomyces* metabolite lactacystin appears to be highly specific (Fenteany *et al.*, 1995).

De-ubiquitinating enzymes. An important step in the ubiquitin pathway involves the release of ubiquitin from its various adducts. Release of ubiquitin plays an essential role in two processes, the first of which is protein degradation. During degradation, it is important to release ubiquitin from Lys residues of end proteolytic products, to disassemble polyubiquitin chains and to 'proofread' mistakenly ubiquitinated proteins. The second process is ubiquitin biosynthesis. Ubiquitin is synthesized in a variety of functionally distinct forms. One of them is a linear, head-to-tail polyubiquitin precursor. Release of the free molecules involves specific enzymatic cleavage between the fused residues. The last ubiquitin moiety in many of these precursors is encoded with an extra C-terminal residue that has to be removed in order to expose the active C-terminal Gly. In a different precursor, ubiquitin is synthesized as an N-terminal fused extension of two ribosomal proteins and serves as a covalent 'chaperone' that targets them to the ribosome. Following their incorporation into the ribosomal complex, ubiquitin is cleaved.

In general, the recycling enzymes are thiol proteases that recognize the C-terminal domain/residue of ubiquitin (reviewed in Hochstrasser, 1996; Wilkinson, 1997). They are divided into two classes: ubiquitin C-terminal hydrolases (UCHs) and ubiquitin-specific proteases (UBPs; isopeptidases). UCHs are ~25 kDa enzymes that are involved in co-translational processing of pro-ubiquitin gene products and in the release of ubiquitin from adducts with small molecules, such as amines and thiol groups. UBPs are ~100 kDa enzymes that catalyze release of ubiquitin from conjugates with cellular proteins or from free polyubiquitin chains. A large number of UBPs are encoded by the yeast genome and higher eukaryotes, suggesting that some of them may have specific functions, such as recognition of distinct tagged substrates. In accordance with the broad spectrum of their functions, they also differ in their characteristics. Some are free, while others are subunits or associated with the 19S complex. Some

require ATP for their activity while others act in an energy-independent manner. Their mechanisms of action also differ, as some are sensitive to ubiquitin aldehyde, while others are not. De-ubiquitinating enzymes can either accelerate proteolysis or inhibit it. By removing ubiquitin moieties from mistakenly tagged proteins, they inhibit proteolysis. Stimulation of proteolysis can be mediated by release of free ubiquitin from biosynthetic precursors and terminal proteolytic products and restoring cellular ubiquitin pool, or by release of ubiquitin from polyubiquitin chains that bind to the 26 proteasome and inhibit it, or by 'editing' polyubiquitin chains and 'fitting' them better for recognition by the 26S proteasome.

Recent experimental evidence indicates that some of these enzymes play an essential role in specific processes and must therefore target specific substrates. The *Drosophila melanogaster FAT FACETS* (*FAF*) gene affects eye development (Huang *et al.*, 1995). Mutant *FAF* flies have more than eight photoreceptors in each of the compound eye units. The protein is probably involved in generating the inhibitory signal sent by the photoreceptor cells to undifferentiated surrounding cells, to stop differentiation and migration to the facet unit. Due to the fact that inactivation of *FAF* can be suppressed by another mutation in a proteasome subunit, it appears that the enzyme stabilizes some unidentified protein(s). A specific serotonin-inducible UCH has been implicated in activation of cAMP-dependent protein kinase A (PKA) in *Aplysia* via stimulation of the degradation of the inhibitory regulatory subunit of the enzyme (Hegde *et al.*, 1997). Degradation is initiated by cAMP that leads to dissociation of the holoenzyme and release of free R subunits. PKA-dependent phosphorylation of a variety of proteins in sensory neurons is responsible for a broad array of morphological changes in the synapse that produce the continuous presynaptic facilitation necessary for long-term behavioral sensitization. UBP3 has been implicated in gene silencing (Moazed and Johnson, 1996). Actively transcribed genes can be silenced following positioning near heterochromatic regions. SIR4 is one *trans*-acting factor that is required for the establishment/maintenance of silencing. One identified SIR4-interacting protein is UBP3, an inhibitor of silencing that acts by either stabilizing an inhibitor or by removing a positive regulator.

Ubiquitin-like proteins

The high evolutionary conservation of ubiquitin enabled the discovery of many ubiquitin-related proteins. Some, such as Parkin, which is implicated in the pathogenesis of certain forms of Parkinson's disease (Kitada *et al.*, 1998), are larger than ubiquitin and possess ubiquitin-like domains that display only slight homology to ubiquitin; they lack the C-terminal Gly and cannot be conjugated. Their physiological significance has remained obscure. A second group contains small proteins with a higher degree of homology to ubiquitin that are involved in post-translational, single or multiple modification of target proteins that serves non-proteolytic purposes (reviewed in Hochstrasser, 1998).

UCRP is an interferon-inducible 15 kDa protein that resembles two tandem repeats of ubiquitin and may be involved in targeting proteins to the cytoskeleton (Loeb and Haas, 1994). Small ubiquitin-related modifier-1 (SUMO-1) is an 11.5 kDa polypeptide involved in targeting RanGAP1 to the nuclear pore complex (NPC) protein RanBP2 (Mahajan *et al.*, 1997). RanBP2 is a GTPase required for the transport of proteins and ribonucleoproteins across the NPC. Its guanosine 5′-triphosphate/diphosphate (GTP/GDP) cycle is regulated by RanGAP1. Localization of RanGAP1 to the NPC is dependent on its single, stable, covalent modification by SUMO-1. The SUMO-1–RanGAP1 conjugate generates a complex with RanBP2 that is essential for the function of RanBP2. SUMO-1 modification of IκBα stabilizes the protein and inhibits NF-κB activation (Desterro *et al.*, 1998). Here, SUMO-1 acts antagonistically to ubiquitin by generating a degradation-resistant protein. SUMO-1 is identical to Sentrin involved in protecting cells against anti-FAS/TNFα-induced apoptosis and, like ubiquitin, can generate multiply modified conjugates with cellular proteins (Kamitani *et al.*, 1997a). NEDD8 is a mammalian protein that is developmentally downregulated and is expressed in high levels in post-mitotic cells characterized by high protein turnover rate, such as skeletal and heart muscle (Kamitani *et al.*, 1997b). RUB1 is a yeast ubiquitin-like protein that was found to modify CDC53/Cullin (Liakopoulos *et al.*, 1998), a common subunit of the SCF ubiquitin ligase complex (see above). While the modification of CDC53 does not affect its stability, it may influence the activity of SCF or its specificity towards its different substrates. Agp12 is another yeast ubiquitin-like protein. Its single, Agp7 (E1)- and Agp10 (E2)-mediated conjugation to Agp5 is essential for autophagy (Mizushima *et al.*, 1998). Conjugation of the ubiquitin-like proteins raises several questions related to the chemical nature of the adduct, the identity of the conjugating enzyme(s) and the specificity of substrate targeting. The C-terminal domain of SUMO-1 is processed proteolytically at residue 97 (TGG^{97}▼H^{98}STV) to generate a free -G^{96}G^{97}-COOH that, like the C-terminal Gly^{76} of ubiquitin, is essential for conjugation. Similarly, RUB1, SMT3 and NEDD8 are also processed to yield a free C-terminal -Gly-Gly. Activation of SMT3 requires at least three proteins: AOS1, UBA2 and UBC9. AOS1 and UBA2 are homologous to the N-terminal and C-terminal domains of E1, respectively, and are probably heterodimerizing to generate an active E1 (Johnson *et al.*, 1997). UBC9 can serve as the E2 in the modification reaction (Schwarz *et al.*, 1998). Conjugation of RUB1 requires ULA1/UBA3 that serve as a heterodimeric E1, and UBC12 as an E2 (Liakopoulos *et al.*, 1998). While conjugation of the known ubiquitin-related proteins does not require E3, it is not clear that this is the case for all of these modifications. The requirement for E3s probably depends on the breadth of spectrum of substrates, and the functions of each of the modifying proteins.

Involvement of the ubiquitin system in the pathogenesis of diseases

Considering the broad range of substrates and processes in which the ubiquitin pathway is involved, it is not surprising that aberrations in the system have been implicated in the pathogenesis of several diseases, both inherited and acquired. The pathological states can be divided into two groups: (i) those that result from loss of function, a

mutation in an enzyme or substrate that leads to stabilization of certain proteins; and (ii) those that result from a gain of function, resulting in accelerated degradation.

Malignancies

It has been noted that the level of p53 is extremely low in uterine cervical carcinomas caused by high-risk strains of HPV. It has been shown that the suppressor is targeted for degradation by E6-AP following formation of a ternary complex with E6-16 or 18, members of the high-risk family of HPV E6 oncoproteins. E6s derived from low-risk strains do not associate with E6-AP and do not destabilize p53 (Scheffner *et al*., 1993; see above). The strong correlation between sensitivity of different genetic polymorphic isotypes of p53 to E6-mediated degradation and the prevalence of cervical carcinoma in women, further corroborates the direct linkage between targeting of p53 and malignant transformation. p53-Arg^{72} is significantly more susceptible to E6 targeting than p53-Pro^{72}. Accordingly, individuals homozygous for the Arg^{72} allele are 7-fold more susceptible to HPV-associated tumors than heterozygotes (Storey *et al*., 1998). Removal of the suppressor by the oncoprotein appears to be a major mechanism utilized by the virus to transform cells. In another case it was shown that c-Jun, but not its transforming counterpart v-Jun, can be ubiquitinated and rapidly degraded. It has been shown that the δ domain of c-Jun, a 27 amino acid sequence that is missing in the retrovirus-derived molecule, destabilizes the protein (Treier *et al*., 1994). This domain is not ubiquitinated but may serve as an anchoring site for the specific E3. The lack of the δ domain from v-Jun, a protein that is otherwise highly homologous to c-Jun, provides a mechanistic explanation for its stability, and possibly for its transforming activity. This is also an example of the complex mechanisms evolved by viruses to ensure continuity of replication and infection. An interesting correlation was found between low levels of p27, the G_1 CDK inhibitor whose degradation is essential for $G_1 \rightarrow S$ transition, and aggressive colorectal (Loda *et al.*, 1997) and breast carcinomas (Catzvaelos *et al.*, 1997). This low level is due to specific activation of the ubiquitin system, as the p27 found in these tumors is the wild type. The strong correlation between the low level of p27 and the aggressiveness of the tumor makes p27 a powerful prognostic tool for survival. Another interesting example involves β-catenin, which plays a major role in signal transduction and differentiation of the colorectal epithelium, and possibly in the multi-step development of the highly prevalent colorectal tumors. In the absence of signaling, glycogen synthase kinase-3 (GSK-3) is active and, via phosphorylation of a specific Ser residue, targets β-catenin for degradation (Aberle *et al*., 1997; Rubinfeld *et al*., 1997). Stimulation promotes dephosphorylation, stabilization and subsequent activation of β-catenin via complex formation with otherwise inactive subunits of transcription regulators such as lymphocyte enhancer factor (LEF) and T-cell factor (TCF). In the cell, β-catenin generates a complex with other proteins, including the tumor suppressor adenomatous polyposis coli (APC). The complex may be analogous to the ligase complexes cyclosome/APC and SCF (see above); here too, the identity of the ligase subunit is unknown.

Genetic diseases

Cystic fibrosis (CF). The CF gene encodes the CF transmembrane regulator (CFTR), which is a chloride ion channel. Only a small fraction of the wild-type protein matures to the cell surface; most of the protein is degraded from the ER by the ubiquitin system (Ward *et al*., 1995). The most frequent mutation in CFTR is ΔF508. Despite normal ion channel function, $CFTR^{\Delta F508}$ does not reach the cell surface at all and is retained in the ER, from which it is degraded. It is possible that the rapid and efficient degradation results in complete lack of cell surface expression of the ΔF508 protein, and contributes to the pathogenesis of the disease.

Angelman's syndrome. This is a rare inherited disorder characterized by mental retardation, seizures, frequent out-of-context laughter and abnormal gait. The syndrome is an example of genomic imprinting and the deleted chromosomal segment is always maternal in origin. The affected protein is the E3 enzyme E6-AP (Kishino *et al*., 1997). While the target substrate of E6-AP has not been identified, elucidation of the defect clearly demonstrates an important role for the ubiquitin system in human brain development. It also shows that E6-AP has a native cellular substrate(s) targeted in the absence of E6.

Liddle syndrome. This is an hereditary form of hypertension that results from deletion of a proline rich (PY) region in the β and γ subunits of the heterotrimeric (αβγ) amiloride-sensitive ENaC. The HECT domain E3 NEDD4 binds to the PY motif of ENaC via its WW domain. ENaC is short-lived *in vivo*, and its α and γ chains were shown to ubiquitinated (Staub *et al*., 1997). Mutations affecting recognition of the channel result in its stabilization, excessive reabsorption of sodium and water, and the subsequent development of hypertension.

Immune and inflammatory responses

Two interesting examples involve an interaction between the ubiquitin pathway and viruses, where the viruses exploit the system to escape immune surveillance. The Epstein–Barr nuclear antigen 1 (EBNA-1) protein persists in healthy carriers for life and is the only viral protein regularly detected in all EBV-associated malignancies. Unlike EBNAs-2, 3 and 4, which are strong immunogens, EBNA-1 cannot elicit a cytotoxic T lymphocyte (CTL) response. The persistence of EBNA-1 contributes, most probably, to some of the virus-related pathologies. A long C-terminal Gly-Ala repeat was found to inhibit degradation of EBNA-1 by the ubiquitin system (Levitskaya *et al*., 1997). Thus, the GA repeat constitutes a *cis*-acting element that inhibits processing and subsequent presentation of the resulting epitopes. A second example involves the human cytomegalovirus (CMV) that encodes two ER resident proteins, US2 and US11. These proteins target MHC class I heavy-chain molecules for degradation. The MHC molecules are normally synthesized on ER-bound ribosomes and transported to the ER. In cells expressing US2 or US11, the MHC molecules are transported in a retrograde manner back to the cytoplasm, deglycosylated and degraded by the proteasome following ubiquitination (Wiertz *et al*., 1996). The viral products bind to the MHC molecules and escort them to the translocation machinery,

where they are transported back into the cytoplasm. The virus-mediated destruction of the MHC molecules does not allow presentation of viral antigenic peptides, thus enabling the virus to evade the immune system.

Neurodegenerative diseases

Ubiquitin immunohistochemistry has revealed enrichment in conjugates in senile plaques, lysosomes, endosomes, and a variety of inclusion bodies and degenerative fibers in many neurodegenerative diseases such as Alzheimer's (AD), Parkinson's and Lewy body diseases, amyotrophic lateral sclerosis (ALS) and Creutzfeld–Jakob disease (CJD) (reviewed in Mayer *et al.*, 1996). However, from these morphological studies it is impossible to conclude what pathogenetic role the ubiquitin system plays in these pathologies. While there can be a cell-specific defect in one of the enzymes of the system, it is more likely that an alteration in one of the protein substrates, either inherited or acquired, renders it resistant to proteolysis. Accumulation of the substrate(s) and/or of the resulting conjugates in aggregates and inclusion bodies may be toxic to the cell. Lack of animal models for most of these diseases and their long periods of development make any mechanistic approach to the problem difficult.

An interesting case involves the proteasome-mediated degradation of the cleaved, C-terminal fragment of presenilin 2 (PS2; Kim *et al.*, 1997). PS2 is a transmembrane protein that is probably involved in trafficking\processing of proteins between different cellular compartments. It is implicated in the transport of the amyloid precursor protein (APP) and its processing to amyloid β42. Mutations in PS2 and in its homologous protein, PS1, are responsible for the majority (>50%) of cases of early onset AD. One mutation, N141I, is prevalent in the Volga-German type of familial AD. For normal functioning, PS2 is first cleaved and the C-terminal domain is degraded. The N-terminal domain probably constitutes the active form of the molecule. Proteasome inhibitors lead to accumulation of polyubiquitinated PS2, and also to accumulation of the C-terminal fragment. Introduction of the Volga-German mutation to wild-type presenilin leads to a dramatic decrease in the rate of processing of PS2, similar to that observed in proteasome inhibitor-treated cells. Thus, it appears that a defect in the processing (and possible subsequent activation) of PS2 may play a role in the pathogenesis of this form of AD. In a different example, a frameshift mutation in the ubiquitin-B gene was identified in a patient with the more prevalent nonfamilial late-onset form of AD (van Leeuwen *et al.*, 1998). While it is clear that the mutation plays an important role in the pathogenesis of the disease, it is possible that a primary, so far unidentified event leads to formation of abnormal protein(s), and the lack of a functional ubiquitin system leads to their accumulation and the resulting pathology.

In Huntington disease and spinocerebellar ataxias, the affected genes, *HUNTINGTIN* and *ATAXINS*, encode proteins with various lengths of CAG/polyglutamine repeats. Recent studies have shown that these proteins aggregate in ubiquitin- and proteasome-positive intranuclear inclusion bodies (Davies *et al.*, 1997; Cummings *et al.*, 1998). It is possible that these abnormal proteins cannot be removed by the system, and their aggregation and precipitation play a role in cell toxicity and subsequent pathologies.

Ubiquitin and muscle wasting

Skeletal muscle wasting, which occurs in various pathological states such as fasting, starvation, sepsis and denervation, results from accelerated ubiquitin-mediated proteolysis (reviewed in Mitch and Goldberg, 1996). The extracellular stimuli and signaling pathways that activate the ubiquitin system in response to the different pathological states are still obscure.

Diseases associated with animal models

Two interesting pathological states have been described in mouse models which may also have implications for human diseases. Inactivation of HR6B, an E2 involved in DNA repair and in targeting of the N-end rule pathway and other protein substrates, leads to the single defect of male sterility due to defects in spermatogenesis. The target substrate proteins may be histones, as their degradation is critical for postmeiotic chromatin remodeling which occurs during spermatogenesis (Roest *et al.*, 1996). Another interesting case is that of the *Itch* locus which encodes a novel E3 enzyme. Defects in the locus result in a variety of syndromes that affect the immune system. Some animals develop inflammatory disease of the large intestine. Others develop a fatal disease characterized by pulmonary interstitial inflammation, alveolar proteinosis, inflammation of the stomach and skin glands that results in severe itching and scarring, and hyperplasia of the lymphoid and hematopoietic cells (Perry *et al.*, 1998). The target protein(s) of the Itch E3 is not known.

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