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The Ubiquitin-Mediated Proteolytic Pathway: Mode of Action and Clinical Implications

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Abstract Proteolysis via the ubiquitin system plays important roles in a variety of basic cellular processes. Among these are regulation of cell cycle and division, modulation of the immune and inflammatory responses, and development and differentiation. In all cases studied, these complex processes are mediated via degradation or processing of a single or a subset of specific proteins. Ubiquitin-mediated degradation of a protein involves two discrete and successive steps: (1) conjugation of multiple moieties of ubiquitin to the protein, and (2) degradation of the conjugated protein by the 26S proteasome complex with the release of free and reutilizable ubiquitin. In a few cases, it has been reported that ubiquitination targets membrane-anchored proteins to degradation in the lysosome/vacuole. An important yet largely unresolved problem involves the mechanisms that endow the system with the high degree specificity and selectivity toward its many substrates. These are determined by a large family of ubiquitin-protein ligases that recognize different primary and/or secondary/post-translational motifs in the different substrates and by a wide array of modifying enzymes, such as protein kinases, and ancillary proteins, such as molecular chaperones, that render them susceptible for recognition by the ligases via modification or association with protein substrates. With the broad spectrum of protein substrates and the complex enzymatic machinery involved in targeting them, it is not surprising that the system was recently implicated in the pathogenesis of several important diseases. In addition, genetic studies in animals underscore the role of the system in normal development. We briefly review the enzymatic cascade involved in ubiquitin-mediated degradation, describe some of the structural motifs identified by the conjugating machinery, and summarize recent developments in the involvement of the system in the pathogenesis of selected disease states. J. Cell. Biochem. Suppl. 34:40–51, 2000.

Key words: ubiquitin; degradation; disease states

Ubiquitin modification of many cellular proteins plays important roles in a variety of basic cellular processes. Among these are regulation of cell cycle and division and of certain aspects of differentiation and development, modulation of the cellular response to stress and extracellular effectors, morphogenesis of neuronal networks, down-regulation of cell surface receptors and ion channels, quality control in the cytosol and the secretory pathway, DNA repair, regulation of the immune and inflammatory responses, and biogenesis of organelles. Although the mechanisms that underlie these complex processes are poorly understood and many of the target proteins have yet to be identified, it is now accepted that, in most cases, modification of the protein substrate by ubiquitin targets it for degradation by the 26S proteasome complex or in the vacuole/lysosome. Hundreds of cellular proteins are known to be targeted by the ubiquitin system, and the list is growing steadily. Among these are cell cycle regulators such as mitotic and G1 cyclins and cyclin-dependent kinase inhibitors, tumor sup-

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pressors such as p53, transcriptional activators and their inhibitors, myc, NF-κB and IκBα, for example, cell surface receptors such as the growth hormone receptor and the T-cell receptor, and endoplasmic reticulum (ER) proteins such as the cystic fibrosis transmembrane conductance regulator (CFTR). Abnormal and otherwise denatured/misfolded proteins are recognized specifically and removed efficiently by the system.

Degradation of a protein via the ubiquitin-proteasome pathway involves two discrete independent and successive steps: (1) generation of a polyubiquitin chain covalently conjugated to the protein substrate, and (2) degradation of the tagged protein by the 26S proteasome (for selected recent reviews on the ubiquitin system, see Hershko and Ciechanover, 1998; Ciechanover, 1998; Baumeister et al., 1998; Ciechanover and Schwartz, 1998; Larsen and Finley, 1997; Pagano, 1997; Hochstrasser, 1996; Coux et al., 1996; Hershko, 1996; Hilt and Wolf, 1996; Wilkinson, 1995; Deshaies, 1995; Jentsch and Schlenker, 1995; Varshavsky, 1996]. Conjugation of ubiquitin to the protein substrate proceeds via a three-step cascade mechanism (Fig. 1). Initially, ubiquitin, a 76-amino acid residues evolutionarily conserved protein, is activated in its C-terminal Gly by the ubiquitin-activating enzyme, E1. After activation, one of several E2 enzymes (ubiquitin-carrier proteins, or ubiquitin-conjugating enzymes [Ubc]) 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 and third step in the conjugation process, covalent attachment of ubiquitin to the substrate. The first moiety is linked to an ϵ-NH_2 group of a Lys residue of the protein substrate to generate an isopeptide

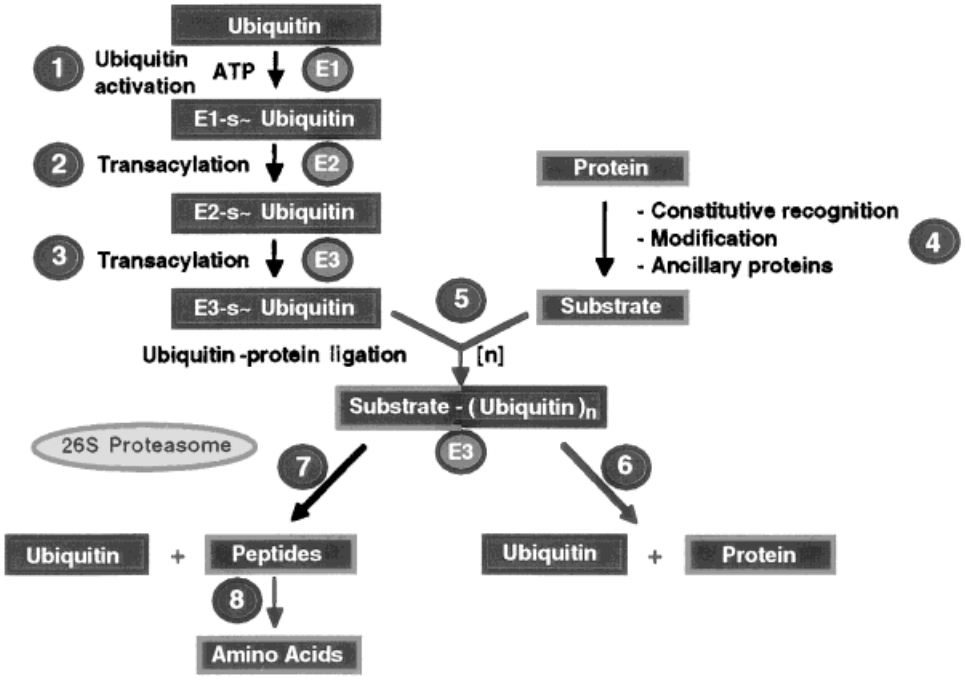

Fig. 1. The ubiquitin-proteasome pathway. 1, Activation of ubiquitin by the ubiquitin-activating enzyme, E1, and formation of an E1-S ~ubiquitin high-energy thiolester intermediate. 2, Transfer, via transacylation, of activated ubiquitin from E1 to a member of the ubiquitin carrier proteins (ubiquitin-conjugating enzymes, Ubc's) family of enzymes, E2. A high-energy E2-S~ubiquitin intermediate is generated. 3, Transfer, via transacylation (in certain cases), of activated ubiquitin from E2 to a member of the ubiquitin-protein ligases family of enzymes, E3. A high-energy E3-S~ubiquitin intermediate is generated in these cases. 4, Conversion of a cellular protein into a substrate of the ubiquitin system. Certain proteins can be recognized directly (constitutive substrates), while others have to undergo a post-translational modification or association with an ancillary protein in order to be targeted. 5, Association of the protein substrate with E3 and processive transfer of activated ubiquitin moieties to generate a polyubiquitin chain anchored to an ϵ-NH_2 group of a Lys residue of the substrate. 6, Removal, via the activity of isopeptidases, of ubiquitin moieties "mistakenly" attached to a protein not destined for degradation. 7, Degradation of the ubiquitin-tagged substrate by the 26S proteasome complex. In steps 6 and 7, free and reutilizable ubiquitin is released. 8, Peptidases-mediated degradation of peptides released by the proteasome to free amino acids.

bond. After binding of the first moiety, a polyubiquitin chain is synthesized by processive transfer of additional activated ubiquitin moieties to Lys^{48} of the previously conjugated ubiquitin molecule. The chain serves, most probably, as a recognition marker for the 26S proteasome. The structure of the ubiquitin system is hierarchical: a single E1 carries out activation of ubiquitin required for all modifications. It transfers ubiquitin to all known species of E2. Each E2 can transfer activated ubiquitin to either a single specific E3 or to several ligases. Although only few E3 enzymes have been identified so far, it is clear that they belong to a large and rapidly growing family of proteins.

A major problem that remains to be resolved involves the mechanisms that underlie the high specificity and selectivity of the system. Why are certain proteins constitutively long-lived, while others are extremely unstable? Why are certain cell cycle regulators degraded in a programmed manner at a particular step of the cell cycle, while they are stable during other phases? Why are certain transcription factors and growth modulators destabilized or stabilized only after a specific extracellular stimulus? Specificity appears to be determined by two distinct and unrelated groups of proteins. Within the ubiquitin system, the substrates are specifically recognized and bound to the different E3 enzymes. Some substrates are recognized via genetically coded primary structural motifs and are degraded constitutively. Many proteins must undergo post-translational modification such as phosphorylation or associate with ancillary proteins such as molecular chaperones in order to be recognized by the appropriate ligase. Thus, the modifying and associating proteins also play major roles in recognition. As for the E3 enzymes, it is unlikely that each targets a single protein substrate. Rather, it is conceivable that a single E3 recognizes a subset of substrates by recognition of similar, but clearly not identical, structural motifs.

After conjugation, the ubiquitin-tagged substrate is degraded by the 26S proteasome complex which is the main proteolytic arm of the ubiquitin pathway. The complex is composed of a core, 20S catalytic subcomplex flanked on both sides by 19S regulatory subcomplexes. With one known exception, ornithine decarboxylase (ODC) that is proteolyzed after association with its inhibitor, antizyme, but without prior ubiquitination, the 26S complex recognizes specifically ubiquitin-tagged proteins. Several recent review articles have described the structure and function of the proteasome complexes in detail (see, e.g., Baumeister et al., 1998; Larsen and Finley, 1997; Coux et al., 1996; Hilt and Wolf, 1996].

An important step in the ubiquitin cycle involves the release of ubiquitin from its various adducts which is catalyzed by ubiquitin-C-terminal specific proteases (UBPs; isopeptidases). Release of ubiquitin plays an essential role mostly in two processes: protein degradation and ubiquitin biosynthesis. During protein degradation, it is important to release ubiquitin from Lys residues of end proteolytic products, to disassemble polyubiquitin chains, to "proofread" mistakenly ubiquitinated proteins, and probably to trim "abnormally" long polyubiquitin chains so that they will be recognized by the ubiquitin "receptor" subunit(s) of the 19S complex. Ubiquitin is synthesized in a variety of functionally distinct forms. One form is a linear, head-to-tail polyubiquitin precursor. In another form, the last ubiquitin residue in some of the polyubiquitin precursor molecules is encoded with an extra C-terminal amino acid residue. In two other precursor forms, ubiquitin is synthesized as a C-terminal fused extension of two ribosomal proteins, probably serving as a covalent "chaperone" that targets these proteins to the ribosome. Release of ubiquitin from all these biosynthetic precursors, which is important in maintaining the free pool of cellular ubiquitin, is catalyzed by specific enzyme(s) that cleave between the C-terminal residue of the upstream moiety and the N-terminal residue of the following moiety.

The high evolutionary conservation of ubiquitin enabled, by molecular and genetic tools, to discover many ubiquitin-related proteins that are expressed in all eukaryotes. Some of these proteins are larger than ubiquitin and contain ubiquitin-like domains. They are not involved in protein modification. Other members of the family have a molecular mass similar to that of ubiquitin, contain a C-terminal Gly residue that can be activated, and have been found to be conjugated to a variety of cellular targets. They serve either as "covalent" chaperones that target the tagged proteins to their subcellular destination or as "antagonists" to ubiquitin. In

general, they generate singly modified adducts that cannot be polyubiquitinated and degraded.

THE UBIQUITIN PATHWAY ENZYMES

The Ubiquitin-Activating Enzyme, E1

A single E1 carries out all ubiquitin modifications in mammalian cells. Deletion of the E1 gene is lethal.

Ubiquitin-Carrier Proteins or Ubiquitin-Conjugating Enzymes (Ubc), E2

Thirteen genes encoding E2 enzymes have been identified in the genome of *Saccharomyces cerevisiae,* and many more have been described in mammalian cells. Some E2 are involved in the degradation of the general population of cellular proteins and have overlapping functions, while others appear to be more specific. *S. cerevisiae* Ubc4 and Ubc5 and their human homologues UbcH5 and UbcH7 are involved in the degradation of many abnormal and short-lived regulatory proteins. Disruption of mouse UbcM4, which is homologous to the yeast Ubc4 and Ubc5, leads to embryonic lethality as a result of general failure in the development of the embryo. As for specific functions of certain E2s, *Drosophila melanogaster* UbcD1 is required for proper detachment of telomeres in mitosis and meiosis, while the *bendless* gene is required for the formation of synaptic networks during development. The disruption of mouse HR6B, one of the mouse homologues of yeast Ubc2/Rad6, results in a specific single defect, male sterility caused by impairment in spermatogenesis (see below).

Because of the specific effects of mutations and deletions in some E2 genes, it has been proposed that these enzymes may participate in direct recognition of the protein substrates. Experimental evidence for direct interaction between E2 and protein substrates is sparse. It appears that catalysis of polyubiquitination that targets proteins for degradation is mediated by E3s to which E2s bind and transfer in some cases, the activated ubiquitin moieties. In other cases, the activated ubiquitin is transferred directly from the E2 to the E3-bound substrate. It is possible that the specific E2-mediated functions are catalyzed by specific E3 that interact on one hand with a specific E2 and on the other hand with a specific proteins substrate or a subset of substrates involved in the development of a defined phenotype.

Ubiquitin-Protein Ligases, E3

Ubiquitin-protein ligases catalyze transfer of activated ubiquitin to the substrate that is bound to them either directly or by an ancillary protein. Whereas E3s play a major role in selection of protein for conjugation and subsequent degradation, the number of the enzymes identified so far is still limited, and little is known of their mode of action. One obstacle to the discovery of novel species of E3s is the lack of sequence homology between the different known enzymes. Also, some E3s are part of large targeting complexes, and the identity of ubiquitin ligating subunit is unknown (see below). Generally, the E3s that have been identified thus far can be classified in four groups [reviewed in Hershko and Ciechanover, 1998; Ciechanover, 1998], although the division may be artificial in many respects:

1. The first group contains the "N-end rule" E3, E3α (Ubr1p in yeast) and E3β. E3α recognizes and binds "N-end rule" protein substrates via their basic (type I) or bulky-hydrophobic (type II) N-terminal amino acid residues. The enzyme has two distinct and independent sites for the two types of the N-terminal residues. Interestingly, it also recognizes, by means of recognition of internal putative "body" sites, non-"N-end rule" proteins. E3α binds to a specific E2 (E2-14 kDa or its yeast homologue Ubc2p/Rad6p), an association that most probably facilitates the transfer of activated ubiquitin to the substrate. E3β is specific for proteins with small and uncharged N-terminal amino acid residues.
2. A second family of E3 enzymes is the HECT (*h*omologous to *E*6-AP *C-terminus*) domain family. The first member of this family, E6-AP (*E6-a*ssociated *p*rotein) is required for human papillomavirus (HPV) E6 oncoprotein-mediated conjugation of p53 (see also below). The action of E6-AP involves formation of a high-energy thiolester with ubiquitin and intramolecular transfer of the activated ubiquitin moiety from the Cys residue to the substrate or the previously conjugated ubiquitin moiety in the polyubiquitin tree. A large family of proteins that contain a C-terminal domain homologous to E6-AP has been described and designated the HECT domain family. All these proteins contain a conserved Cys residue near the C-terminus

that serves as a ubiquitin acceptor. The N-terminal domain is variable among members of the family, most probably serving as a recognition domain for the different protein substrates. As for the functions of the members of the family, it has been shown that mutations in E6-AP result in Angelman's syndrome, an hereditary disease characterized by mental retardation and disturbed gait (see below). This finding suggests that E6-AP-mediated protein ubiquitination is required for brain development and that E6-AP targets certain native cellular proteins in the absence of E6. Interestingly, in yeast, certain members of the HECT domain family are involved in catabolic inactivation of membrane proteins and targeting them for degradation in the vacuole. For example, Npi1 targets Gap1p, the amino acid permease of yeast, for degradation after the addition of NH_4^+ ions. Ubiquitination leads to endocytosis of the membrane proteins and to its subsequent targeting to the vacuole. An interesting motif common to many members of the HECT domain family of enzymes is the WW domain, an ~30-amino acid region most probably involved in interactions with Pro-rich (XPPXY or "PY") motifs in the target substrates. One mammalian enzyme that contains several WW domains is Nedd4 that targets the epithelial sodium channel, ENaC. Mutation(s) in the PY C-terminal motif in ENaC lead(s) to Liddle's syndrome, a form of hypertension caused by the stabilization and consequent accumulation of the sodium channel (see below).

3. A third class of E3 enzymes are the multisubunit complexes involved in degradation of cyclins. The best studied complex, the cyclosome or *a*naphase *p*romoting *c*omplex (APC) has a ubiquitin ligase activity specific for cell cycle regulatory proteins that contain a 9-amino acid proteolytic signal, the "destruction box" (see below). Its known substrates are mitotic cyclins, certain anaphase inhibitors, and spindle-associated proteins, all of which are degraded at the end of mitosis. APC is inactive in the interphase, but it becomes active, probably as a result of phosphorylation, at the end of mitosis. The *Xenopus* complex has eight subunits. The complex acts with a specific E2 partner, E2-C; however, its ubiquitin-ligating subunit has not been identified.
4. The fourth class of ubiquitin ligases are also complexes involved in the degradation of certain other cell cycle regulators, such as the Sic1 CDK inhibitor or the G1 cyclin Cln2. The mammalian complexes have been designated SCF (Skp1p, Cullin, F-box protein) complexes [reviewed in Koepp et al., 1999]. Here, phosphorylation of the substrate converts it to a form susceptible to the action of the ligase complex. Several such ubiquitin ligase complexes, designated SCFs, have been described that share some common subunits, but contain probably distinct subunits specific for certain protein substrates. Degradation of the CDK inhibitor Sic1, which is essential for the G1 → S transition in yeast, requires its phosphorylation by a G1 cyclin-activated protein kinase as well as the products of *CDC34*, *CDC53*, *CDC4*, and *SKP1* genes. Cdc34 is an E2 protein. Cdc53 (the yeast homolog of cullin), Cdc4 (the F-box protein), and Skp1p generate a complex that is responsible for the ubiquitination of phosphorylated Sic1. The Cdc4 (the F-box protein) is most probably, the substrate binding unit. Ubiquitination and degradation of the yeast G1 cyclin Cln2 also requires its phosphorylation and the action of Cdc34, Cdc53, and Skp1, but not of Cdc4. A different F-box protein is involved in this process.

In addition to the four distinct types/families of ubiquitin ligases, several other E3s have been partially characterized. These are involved in the targeting of N-myc, c-Fos, and certain muscle proteins and in the limited processing of the p105 precursor of the transcriptional activator NF-κB (see below). Better characterization is required for their further classification.

UBIQUITIN CONJUGATE-DEGRADING ENZYMES

The 20S and 26S Proteasome Complexes

The eukaryotic (yeast) 20S complex is arranged as a stack of four rings, two α rings that flank two β rings. Each of the different rings contains seven distinct subunits and the complex has a general structure of $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$. The crystal structure [Groll et al., 1997] has shown that three distinct catalytic sites—the trypsin-, chymotrypsin-, and postglutamyl peptidyl hydrolytic (PGPH; post-acidic)-like sites—reside in different β-subunits, generated topo-

logically by adjacent pairs of identical β-type subunits residing in different β rings. The α chains, although catalytically inactive, play an essential role in stabilizing the two-ring structure of the β chains. Also, they probably play a role in the binding of the 19S "cap" regulatory subcomplexes. The crystal structure has also exhibited a distance of 28 Å between the active sites of adjacent active β-subunits. This distance may determine the length of the peptides generated during the proteolytic process (~8-amino acid residues) and may explain the role of the proteasome in generation of antigenic peptides presented subsequently on class I MHC molecules.

Substrate recognition by the 26S proteasome is probably mediated by the interaction of specific subunits of the 19S regulatory complex with the substrate-anchored polyubiquitin chain. The yeast 19S subcomplex can be dissociated into a base subcomplex that associates directly with the 20S proteolytic core subparticle and a lid subcomplex. It has been shown that the lid is required for degradation of polyubiquitinated proteins [Glickman et al., 1998]. A polyubiquitin binding subunit has been described in the 19S subcomplex [S5a in mammals, Mbp1 in plants, and Mcb1(Rpn10p) in yeast]; however, its role as the ubiquitin receptor subunit could not be confirmed in gene inactivation experiments.

Besides the 19S complex, an additional complex that associates with the 20S proteasome and enhances dramatically its activity is PA28 (REG or the 11S regulator). PA28-20S-PA28 complex does not degrade ubiquitinated proteins. After association, PA28 increases the efficiency of the 20S complex toward a whole array of different peptides. Since the activator is induced by interferon-γ (IFN-γ), it was suggested that it plays a role in the antigen processing function of the proteasome by trimming large peptides that were generated by the 26S complex to the precise antigenic epitopes recognized by the class I MHC complex and the appropriate CTLs. It was suggested that a 19S-20S-PA28 mixed complex can successively carry out all the immune system-related functions of the proteasome [Hendil et al., 1998].

An unresolved problem involves the entry of protein substrates and the exit of proteolysis products from the proteasome. In the *Thermoplasma* proteasome, there are two entry pores at the two ends of the cylinder. In the yeast 20S proteasome, the N-terminal domains of the α subunits protrude toward each other and fill the space. Thus, entry from the ends may be possible only after substantial rearrangement that may occur after association with the 19S subcomplexes. It should be noted, however, that the yeast complex has 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 apertures through which unfolded and extended protein substrates may enter.

An important development involves the synthesis and discovery of proteasome inhibitors. Specific inhibitors of the proteasome are powerful research tools that have the potential to serve as drugs under certain circumstances when transient modulation of the ubiquitin system activity is needed (see below). Interestingly, under basal metabolic conditions, in which lysosomal degradation is negligible, they inhibit the degradation of the bulk of cellular short- and long-lived alike. This finding suggests that the ubiquitin system has a major role in cellular proteolysis. An interesting and specific proteasomal inhibitor is the *Streptomyces* metabolite lactacystin [Fenteany et al., 1995]. It modifies covalently the active site Thr^1 residue and strongly inhibits the trypsin- and chymotrypsin-like activities of the complex.

De-ubiquitinating Enzymes

In general, the recycling enzymes are thiol proteases that recognize specifically the C-terminal domain of ubiquitin [Hochstrasser, 1996; Wilkinson, 1995]. They are generally divided into two classes: ubiquitin C-terminal hydrolases (UCHs) and UBiquitin-specific proteases (UBPs; also called isopeptidases). The UCHs are ~25.0-kDa enzymes most probably involved in co-translational processing of pro-ubiquitin gene products and in the release of ubiquitin from adducts with small molecules. The UBPs are ~100.0-kDa enzymes that catalyze release of ubiquitin from conjugates with cellular proteins or from free polyubiquitin chains. Genes of 16 different UBPs are found in the yeast genome, and the number of mammalian UBPs is probably more then twofold larger. This large number suggests that some of the enzymes may have specific functions, such as recognition of conjugates of distinct tagged substrates. Some of the enzymes are free, while others are subunits of or associated with the 19S proteasome complex. Some require ATP for

their activity, whereas others act in an energy-independent manner. Their mechanism of action also differ, as some are sensitive to ubiquitin aldehyde, while others are not. In general, de-ubiquitinating proteins can either accelerate proteolysis or inhibit it, depending on the step in the pathway in which they are acting. 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, terminal proteolytic products, or polyubiquitin chains that bind to the 26 proteasome and inhibit its activity, or by "editing" polyubiquitin chains and "fitting" them better for recognition by the 26S proteasome.

UBIQUITIN-LIKE PROTEINS

As noted above, there are two classes of ubiquitin-like proteins. Some of these proteins, 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 have ubiquitin-like domains that show only slight homology to ubiquitin. They lack the C-terminal Gly and consequently cannot be activated and conjugated to other proteins. The physiological role of the ubiquitin-like domains of these proteins remains obscure. A second group of ubiquitin-like proteins contain smaller proteins with a higher degree of homology to ubiquitin that are involved in post-translational, single or multiple, covalent modification of target proteins that serve, most probably, nonproteolytic purposes [reviewed by Hochstrasser, 1998].

One interesting and well-studied small ubiquitin-like protein is SUMO1, which is involved, among other functions, in targeting RanGAP1 to the nuclear pore complex (NPC) protein RanBP2. RanBP2 is a small Ras-like GTPase required for the transport of proteins and RNPs across the NPC. An important regulator of the GTP/GDP cycle is the Ran GTPase-activating protein RanGAP1. Localization of RanGAP1 to the NPC is dependent on its covalent stable modification by a single moiety of SUMO1. SUMO1 · RanGAP1 conjugate generates a complex with RanBP2 that is essential for the function of RanBP2 in the NPC. Another important function of SUMO-1 appears to be modulation of the proteolytic-signaling activity of ubiquitin: conjugation of SUMO-1 to IκBα stabilizes the protein and prevents its phosphorylation-dependent ubiquitination and degradation. Here, SUMO-1 acts antagonistically to ubiquitin as a negative regulator of degradation [Desterro et al., 1998].

Conjugation of the ubiquitin-like proteins raises several questions such as the chemical nature of the adduct, the identity of the conjugating enzyme(s), and the specificity of substrate targeting. All known ubiquitin-like proteins involved in conjugation contain a C-terminal Gly residue that is essential for conjugation and that in many cases is exposed only following post-translational processing. Activation of these proteins requires at least three proteins: two that are homologous to the N-terminal and C-terminal domains of E1 and that heterodimerize to generate and active E1, and an E2-like enzyme. These enzymes cannot activate ubiquitin. Whether E3s are also involved in the process is unclear. Their possible involvement will probably depend on the spectrum of substrates of each of the different modifying proteins.

UBIQUITINATION TARGETING SIGNALS

As noted, an important, yet largely unresolved, problem involves the identity of the structural signals that target the myriad substrates of the system for conjugation and subsequent degradation and that determine its high specificity and selectivity. Some primary signals are recognized directly and constitutively by E3s. However, regulated degradation involves acquirement of secondary, post-translational signals or association with ancillary proteins.

The first signal that has been identified on a model protein is the N-terminal residue ("N-end rule" pathway) [Varshavsky, 1996]. However, further studies have shown that this recognition signal is of extremely limited scope.

A second identified signal is phosphorylation. It was proposed that PEST elements, that is, sequences rich in Pro (P), Glu (E), Ser (S), and Thr (T) residues, undergo phosphorylation by several protein kinases, and it is the post-translational modification that targets the proteins for conjugation and subsequent degradation. Degradation of the yeast G1 cyclins, for example, is mediated by multiple phosphorylations within the proteins' PEST sequences. IκBα, the inhibitor of the transcriptional factor NF-κB, is degraded after extracellular stimulation-induced phosphorylation on two specific

Ser residues, 32 and 36. Degradation of the inhibitor exposes a nuclear localization signal on the heterodimeric NF-κB, enabling its translocation into the nucleus, where it activates specific transcription. It has been shown that the phosphorylated domain serves as a direct recognition and binding site for the E3, and that inhibition of the E3, by mimetic peptides that span this domain inhibit the biological functions of NF-κB. It was noted that β-catenin, which is also targeted for ubiquitin-mediated degradation by phosphorylation, has a sequence motif similar to that of IκBα (see below). Recent findings indicate that both proteins are targeted by the same E3 complex SCF [reviewed by Koepp et al., 1999; Laney and Hochstrasser, 1999]. In other cases, protein phosphorylation prevents degradation. For example, phosphorylation of the c-mos, c-fos, and c-jun proto-oncogenes by MAP kinases suppresses their ubiquitination and degradation.

A third important targeting signal is the "destruction box" of mitotic cyclins and certain other cell cycle regulators. The box is a partially conserved 9-amino acid long sequence typically located in the N-terminal domain of the target molecule. It is both necessary and sufficient for their ubiquitination and subsequent degradation. The general sequence of the box is: R-(A/T)-(A)-L-(G)-X-(I/V)-(G/T)-(N). R and L in positions 1 and 4 are indispensable, while the remaining bracketed residues appear in most of the known "boxes." Some proteins that are unrelated to cell cycle regulation, such as the yeast uracil permease, are also targeted in a "destruction box"-dependent manner, but it is unknown whether the cyclosome/APC is involved in this process. The mechanism of targeting by the "destruction" box remains enigmatic. It does not involve phosphorylation, as mutations in the Ser or Thr residue do not alter its function. Also, since it does not contain a Lys residue, it does not serve as a ubiquitination site. It may serve as binding site for the ligase subunit of the E3.

Other recognition domains have also been described, but they are less well defined. The δ-domain in c-Jun is a 27-amino acid residue sequence in the N-terminal domain that has been shown to be a transferable "destabilization" signal (see below). The α factor receptor of yeast, Ste2, is a G-protein-coupled signal transducing receptor that binds the α factor mating pheromone. Ligand binding leads to ubiquitination mediated by a 9-amino acid SINNDAKSS motif. Interestingly, mono-ubiquitination is sufficient to promote internalization. After ubiquitination, the receptor is internalized and transported to the vacuole, where it is degraded. Iron down-regulates the iron regulatory protein-1 (IRP-1), a process that requires the participation of a cluster of Cys residues at the N-terminal domain. It was proposed that changes in the oxidation state of the bound iron leads to oxidation of certain amino acid residues in the iron binding domain, which are then recognized by the ubiquitin ligase. An interesting case involves the processing of p105, the precursor molecules of the NF-κB subunits p50. p50 is derived from the N-terminal domain of the precursor molecule, while the C-terminal domain is degraded. The process is mediated by the ubiquitin system, and it is the only known case in which the ubiquitin system is involved in limited processing, rather than in complete destruction of the target substrate. It has been recently shown that a Gly-rich region (GRR) that spans amino acid residues 376–404 and that contains 19 (out of 29) Gly residues constitutes an independent transferable "stop signal" that prevents processing of p105. Mechanistically, GRR interferes with the action of the 26S proteasome and does not affect recognition by the conjugating enzymes (see also below).

INVOLVEMENT OF THE UBIQUITIN SYSTEM IN THE PATHOGENESIS OF DISEASES

With the broad range of ubiquitin-targeted substrates and the complexity of the enzymatic cascade involved, it is not surprising that aberrations in the process have recently been implicated in the pathogenesis of several diseases, both inherited and acquired. The pathological states can be divided into two major groups: (1) those that result from loss of function—mutations in an enzymatic component or a target substrate that result in stabilization of certain proteins; and (2) those that result from gain of function, accompanied by abnormal accelerated degradation of the protein target(s).

MALIGNANCIES

It was noted that the level of the tumor suppressor protein p53 is extremely low in uterine cervical carcinoma tumors caused by high-risk strains of human papillomavirus (HPV). Detailed mechanistic studies have shown that the suppressor is targeted for ubiquitin-mediated

degradation by the high-risk species of the HPV oncoprotein E6. Further corroborating the linkage between targeting of the suppressor for degradation and malignant transformation is the correlation between sensitivity of different isotypes of p53 to E6-mediated degradation and the prevalence of cervical carcinoma in human carriers of these isotypes [Storey et al., 1998], though this correlation still requires further corroboration, see for example Helland et al. [1998]. Removal of the suppressor by the oncoprotein is assumed to be a key mechanism used by the virus to transform cells. E6-dependent degradation is mediated by E6-AP E3 and by its interacting E2 UbcH8. E6 serves as an ancillary protein that associates with both the ligase and the target substrate. By the generation of a ternary complex that brings them into the required proximity, it probably enables catalysis of conjugation.

In another case, it was shown that c-Jun, but not its transforming counterpart, v-Jun, can be multiply ubiquitinated and rapidly degraded in cells. The differential sensitivity to the ubiquitin system is due to the δ domain of c-Jun, an amino acid sequence that spans residues 31–57 and is not present in the retrovirus-derived molecule. Mutational deletion of the domain stabilizes c-Jun. The lack of the δ domain from v-Jun, a protein that is otherwise highly homologous to c-Jun, provides a mechanistic explanation for the stability, and possibly the resulting transforming activity, of v-Jun.

β-Catenin plays a major role in signal transduction and differentiation of the colorectal epithelium; aberrations in its catabolism are involved in the multi-step development of colorectal tumors. These tumors develop in 50% of the Western world's population by the age of 70; in 10% of these individuals (5% of the population), they progress to malignancy. In the absence of signaling, glycogen synthase kinase-3 (GSK-3) is active and, via phosphorylation, promotes ubiquitin-mediated degradation of β-catenin. Stimulation of cells promotes dephosphorylation, stabilization, and subsequent activation of β-catenin via complex formation with otherwise inactive subunits of other transcription factors and their translocation into the nucleus. In the cell, β-catenin generates a complex that contains, among other components, the 300-kDa tumor suppressor adenomatous polyposis coli (APC), axin and axil, which appears to regulate its intracellular level, in a manner that remains unknown. In colon cancer cells that do not express APC or that harbor APC proteins that are mutated in one of the catenin binding clusters, this association does not occur. Consequently, the protein accumulates as an active transcriptional complex. Expression of full-length APC in these cells leads to degradation of excess β-catenin and to abrogation of the *trans*-activation effect. The ligase that targets β-catenin is an SCF complex, similar to the one that targets IκBα (see above).

An interesting correlation was found between low levels of p27, the G1 cyclin CDK inhibitor, and aggressive colorectal [Loda et al., 1997] and breast [Catzavaelos et al., 1997] carcinomas. Degradation of the protein by the ubiquitin system is essential for G1 → S transition. The low level is caused by up-regulation of an unidentified rate-limiting enzymatic component of the ubiquitin system, as the p27 found in these tumors is the WT species, and not an unstable mutant protein. The strong correlation between the low level of p27 and the aggressiveness of the tumor makes p27 a powerful independent prognostic tool for survival in both tumors.

GENETIC DISEASES

Cystic Fibrosis

Cystic fibrosis (CF) is a common autosomal recessive inherited multisystem disorder of children and adults, characterized by chronic obstruction and infection of airways, and maldigestion with all its consequences. The gene encodes the CF transmembrane regulator (CFTR), which is an epithelial cell surface chloride channel. The most frequent mutation in the gene (~70%) involves deletion of phenylalanine 508 (ΔF508). Despite normal ion channel function, $CFTR^{\Delta F508}$ does not reach the cell surface but is retained in the endoplasmic reticulum from which it is degraded by the ubiquitin proteasome pathway. It is possible that the rapid degradation and complete lack of cell surface expression of the ΔF508 protein (and possibly other mutants from which only a small fraction matures to the cell surface and that display a wide spectrum of clinical symptoms) contributes to the pathogenesis of the disease.

Angelman's Syndrome

Angelman's syndrome is a rare inherited disorder characterized by mental retardation, sei-

zures, out-of-context frequent smiling and laughter, and abnormal gait. The syndrome is an example of genomic imprinting, in which phenotypic expression depends on the parent of origin for certain genes. In Angelman's syndrome, the deleted chromosomal segment, 15q11–13, is always maternal in origin. The ubiquitin protein ligase E6-AP was localized within this region and truncated mutants of E6-AP were identified in patients with Angelman's syndrome. While the target protein of the E3 enzyme has not been identified, the elucidation of the defect at the molecular level demonstrates an important role for the ubiquitin system and for E6-AP in human brain development. It also shows that E6-AP has native cellular substrate(s) that are targeted in the absence of E6.

Liddle's Syndrome

Liddle syndrome is a rare hereditary form of hypertension which results from deletion of the proline rich (PY) regions of the β- and γ-subunits of the amiloride-sensitive epithelial sodium channel (ENaC), leading to hyperactivity of the channel (see above). Via its WW domain, the ubiquitin ligase Nedd4 binds to the PY motif of ENaC. ENaC is a short-lived complex that is targeted, after ubiquitination, to degradation in the lysosome. Mutations that affect recognition/processing via this pathway result in stabilization of the channel, excessive reabsorption of sodium and water, and subsequent development of hypertension.

IMMUNE AND INFLAMMATORY RESPONSES

Peptides epitopes presented to cytotoxic T cells (CTLs) on class I MHC molecules are generated in the cytosol by ubiquitin- and proteasome-mediated limited processing of antigenic proteins. The cytokine interferon-γ (IFN-γ) induces three 20S proteasomal subunits that exchange with existing subunits. This substitution alters the cleavage site preferences of the proteasome and results in peptides that terminate predominantly in basic and hydrophobic residues. Such peptides are similar to the vast majority of known peptides presented on class I MHC molecules and are probably better recognized by the TCR. IFN-γ also induces PA28 that may accelerate generation of antigenic peptides from larger proteolytic products derived from intact proteins (see above). Under "normal" conditions, the ubiquitin system degrades, in a nondiscriminatory manner, both intracellular "self" proteins, as well as foreign, "non-self" proteins. Peptides from both populations are presented to CTLs, but those derived from "self" proteins do not elicit a T-cell response. It is possible that aberrations in processing of these proteins may lead to presentation of mistakenly processed "self" peptides as "non-self." This can serve as the pathogenetic basis for a whole array of autoimmune diseases.

Many immune and inflammatory disorders can also be elicited by untoward activation of the immune system's major transcription factor NF-κB that is mediated by the ubiquitin system (see above). Activation of the factor leads to increased transcription of many cytokines, adhesion molecules, inflammatory response and stress proteins, and immune system receptors.

Two interesting examples involve an interaction of the ubiquitin pathway and viruses, where the viruses exploit the system to escape immune surveillance. Epstein-Barr nuclear antigen-1 (EBNA-1) protein persists in healthy virus carriers for life and is the only viral protein regularly detected in all EBV-associated malignancies. Unlike EBNA 2–4, which are strong immunogens, EBNA-1 is not processed and cannot elicit a CTL response. The persistence of EBNA-1, most probably, contributes to some of the pathologies caused by the virus. An interesting structural feature common to all species of EBNA-1 proteins derived from different EBV strains is a relatively long Gly-Ala repeat at the C-terminal domain of the molecule. This repeat inhibits processing of the conjugated protein by the 26S proteasome, similar to the inhibition conferred by the GRR on the p105 precursor protein of NF-κB (see above). Thus, the Gly-Ala repeat constitutes a *cis*-acting element that inhibits antigen processing and subsequent presentation of the resulting antigenic epitopes. A second example involves the human cytomegalovirus (CMV) that encodes two ER resident proteins, US2 and US11. These proteins down regulate the expression of MHC class I heavy chain molecules. The MHC molecules are normally synthesized on ER-bound ribosomes and transported co-translationally to the ER, where glycosylation occurs. In cells expressing US2 or US11, these proteins are transported in a retrograde manner back into the cytoplasm, deglycosylated, and degraded by the proteasome after ubiquitination. The viral products appear to bind to the MHC molecules and escort them to

the translocation machinery, where they are translocated back into the cytoplasm. By depriving the cells from MHC molecules, the US proteins inhibit presentation of virus-derived antigenic peptides, thus enabling the virus to evade the immune system and replicate.

NEURODEGENERATIVE DISEASES

Accumulation of ubiquitin conjugates has been reported in the pathologic lesions of many chronic neurodegenerative diseases, such as the neurofibrillary tangles of Alzheimer's disease and brainstem Lewy bodies in Parkinson's disease. Accumulation in many of these cases appears to be secondary and may reflect attempts by the cell to remove damaged or abnormal proteins. Recently, a frameshift mutation in the ubiquitin-B transcript was noted that may be one cause of the more prevalent nonfamilial late-onset form of Alzheimer's disease [van Leeuwen et al., 1998]. Although it is clear that the mutation plays a role in the pathogenesis of the disease, it is possible that a primary, still unidentified event, leads to formation of abnormal protein(s), and the lack of a functional ubiquitin system leads to their accumulation and the resultant pathology. In Huntington disease or in spinocerebellar ataxia types 1 and 3, the affected genes, *HUNTINGTIN* and *ATAXIN 1* and *3*, encode for proteins with various lengths of CAG/polyglutamine repeat expansions. Recent studies have shown that these proteins aggregate in ubiquitin and proteasome positive intranuclear inclusion bodies [Cummings et al., 1998; Davies et al., 1997; Paulson et al., 1997]. Although it is not clear whether these abnormal proteins are targeted by the ubiquitin system, it is possible that this accumulation reflects the inability of the cells to remove the excess of conjugated abnormal protein. Their aggregation and precipitation in intranuclear inclusion bodies, or even accumulation in a soluble form may play a role in cell toxicity and in the subsequent pathogenesis of the diseases.

UBIQUITIN AND MUSCLE WASTING

Skeletal muscle wasting that occurs in various pathological states, such as fasting, starvation, sepsis, and denervation, results from accelerated proteolysis via the ubiquitin pathway. Several studies have demonstrated a close correlation between accelerated rates of proteolysis in skeletal muscle occurring in these pathological conditions, increased levels of mRNA coding for different components of the ubiquitin system, and ubiquitin-protein conjugates. Administration of inhibitors of the proteasome was found to block enhanced muscle proteolysis associated with denervation or sepsis. Thus, the enhanced proteolysis and atrophy of muscle in various pathological states appears to be due primarily to activation of the ubiquitin proteasome pathway.

DISEASES ASSOCIATED WITH ANIMAL MODELS

Two interesting pathological states have been described in animal models that may also have implications for human disease. Inactivation of HR6B, an E2 involved in DNA repair, and in targeting of proteins via the "N-end rule" (see above), leads to a single isolated defect, male sterility, that is associated with defects in spermatogenesis. The target substrate proteins may be histones, as their degradation is critical for postmeiotic chromatin remodeling during spermatogenesis. Another example is that of the *Itch* locus in mice, which encodes for a novel E3 enzyme. Under different genetic backgrounds, defects in the locus result in a variety of syndromes that affect the immune system. Some develop inflammatory disease of the large intestine. Others develop a fatal disease characterized by severe inflammation of the pulmonary interstitium, stomach, and skin glands that results in severe, constant itching and scarring, as well as hyperplasia of the lymphoid and hematopoietic cells.

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