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THE UBIQUITIN SYSTEM

Avram Hershko and Aaron Ciechanover
Unit of Biochemistry, Faculty of Medicine and the Rappaport Institute for Research in the Medical Sciences, Technion-Israel Institute of Technology, Haifa 31096, Israel

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ABSTRACT

The selective degradation of many short-lived proteins in eukaryotic cells is carried out by the ubiquitin system. In this pathway, proteins are targeted for degradation by covalent ligation to ubiquitin, a highly conserved small protein. Ubiquitin-mediated degradation of regulatory proteins plays important roles in the control of numerous processes, including cell-cycle progression, signal transduction, transcriptional regulation, receptor down-regulation, and endocytosis. The ubiquitin system has been implicated in the immune response, development, and programmed cell death. Abnormalities in ubiquitin-mediated processes have been shown to cause pathological conditions, including malignant transformation. In this review we discuss recent information on functions and mechanisms of the ubiquitin system. Since the selectivity of protein degradation is determined mainly at the stage of ligation to ubiquitin, special attention is focused on what we know, and would like to know, about the mode of action of ubiquitin-protein ligation systems and about signals in proteins recognized by these systems.

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INTRODUCTION

The past few years have witnessed a dramatic increase in our knowledge of the important functions of ubiquitin-mediated protein degradation in basic biological processes. The selective and programmed degradation of cell-cycle regulatory proteins, such as cyclins, inhibitors of cyclin-dependent kinases, and anaphase inhibitors are essential events in cell-cycle progression. Cell growth and proliferation are further controlled by ubiquitin-mediated degradation of tumor suppressors, protooncogenes, and components of signal transduction systems. The rapid degradation of numerous transcriptional regulators is involved in a variety of signal transduction processes and responses to environmental cues. The ubiquitin system is clearly involved in endocytosis and downregulation of receptors and transporters, as well as in the degradation of resident or abnormal proteins in the endoplasmic reticulum. There are strong indications for roles of the ubiquitin system in development and apoptosis, although the target proteins involved in these cases have not been identified. Dysfunction in several ubiquitin-mediated processes causes pathological conditions, including malignant transformation.

The role of ubiquitin in protein degradation was discovered and the main enzymatic reactions of this system elucidated in biochemical studies in a cell-free system from reticulocytes (reviewed in 1). In this system, proteins are targeted for degradation by covalent ligation to ubiquitin, a 76-amino-acid-residue protein. The biochemical steps in the ubiquitin pathway have been reviewed previously (2, 3) and are illustrated in Figure 1*A*. Briefly, ubiquitin-protein ligation requires the sequential action of three enzymes. The C-terminal Gly residue of ubiquitin is activated in an ATP-requiring step by a specific activating enzyme, E1 (Step 1). This step consists of an intermediate formation of ubiquitin adenylate, with the release of PP_i, followed by the binding of ubiquitin to a Cys residue of E1 in a thiolester linkage, with the release of AMP. Activated ubiquitin is next transferred to an active site Cys residue of a ubiquitin-carrier protein, E2 (Step 2). In the third step catalyzed by a ubiquitin-protein ligase or E3 enzyme, ubiquitin is linked by its C-terminus in an amide isopeptide linkage to an ε-amino group of the substrate protein's Lys residues (Figure 1*A*, Step 3).

Usually there is a single E1, but there are many species of E2s and multiple families of E3s or E3 multiprotein complexes (see below). Specific E3s appear to be responsible mainly for the selectivity of ubiquitin-protein ligation (and, thus, of protein degradation). They do so by binding specific protein substrates that contain specific recognition signals. In some cases, binding of the

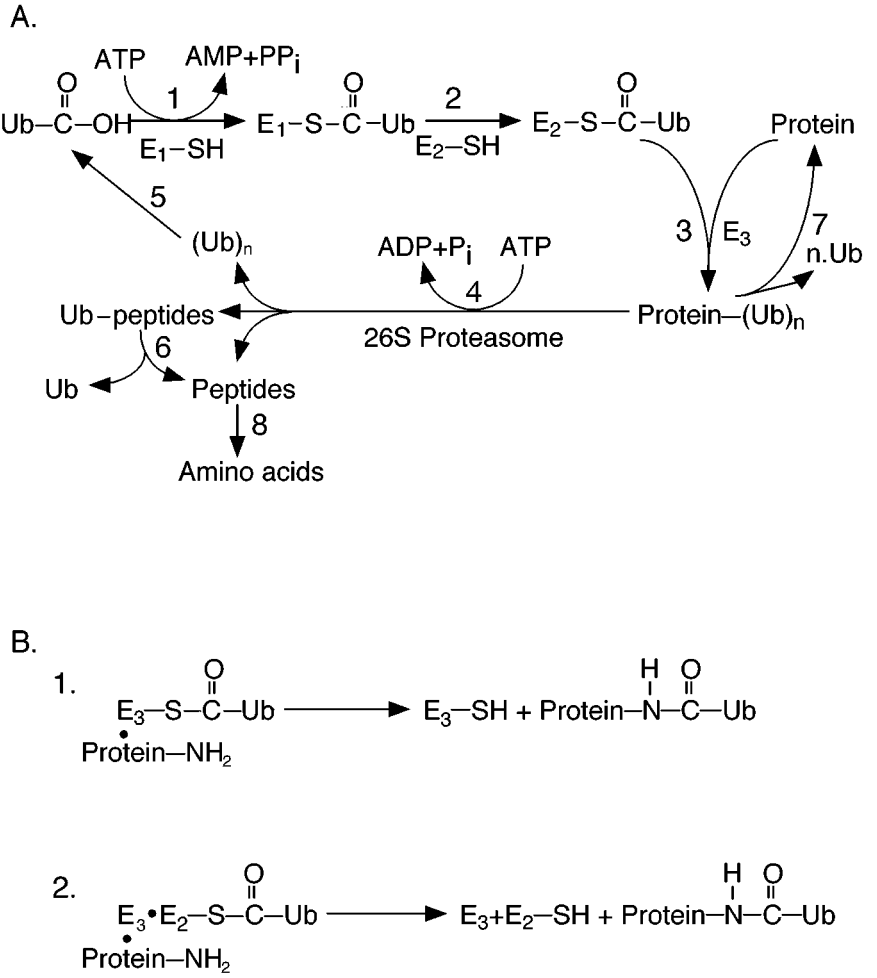

Figure 1 Enzymatic reactions of the ubiquitin system. *A.* Sequence of reactions in the proteolytic pathway. *B.* Possible mechanisms of ubiquitin transfer by different types of E3 enzymes. See the text. *Ub*, ubiquitin.

substrate protein to an E3 is indirect, via an adaptor protein. Different types of E3s may carry out the transfer of ubiquitin to the substrate protein by two different mechanisms. In some cases, such as with the *Hect*-domain family of E3 enzymes (see below), ubiquitin is first transferred from an appropriate E2 to an active site Cys residue of the E3 enzyme. This E3-ubiquitin thiolester is the donor for amide bond formation with the protein substrate (Figure 1*B*.1). In other families of E3 enzymes, E3-ubiquitin thiolester formation cannot be

demonstrated. Since E3 enzymes bind cognate E2s tightly (see below) and they also bind their appropriate protein substrate, ubiquitin can be transferred directly from E2 to the protein substrate (Figure 1*B*.2). After the linkage of ubiquitin to the substrate protein, a polyubiquitin chain is usually formed, in which the C-terminus of each ubiquitin unit is linked to a specific Lys residue (most commonly Lys^{48}) of the previous ubiquitin.

Proteins ligated to polyubiquitin chains are usually degraded by the 26S proteasome complex (reviewed in 4) that requires ATP hydrolysis for its action. The 26S proteasome is formed by an ATP-dependent assembly of a 20S proteasome, a complex that contains the protease catalytic sites, with 19S "cap" or regulatory complexes (5). The 19S complexes contain several ATPase subunits and other subunits that are presumably involved in the specific action of the 26S proteasome on ubiquitinylated proteins. The roles of ATP in the assembly of the 26S proteasome complex and in its proteolytic action are not understood. The action of the 26S proteasome presumably generates several types of products: free peptides, short peptides still linked to ubiquitin via their Lys residues, and polyubiquitin chains (Figure 1*A*, Step 4). The latter two products are converted to free and reusable ubiquitin by the action of ubiquitin-C-terminal hydrolases or isopeptidases (Steps 5 and 6). Some isopeptidases may also disassemble certain ubiquitin-protein conjugates (Step 7) and thus prevent their proteolysis by the 26S proteasome (see below). The latter type of isopeptidase action may have a correction function to salvage incorrectly ubiquitinylated proteins or may have a regulatory role. Short peptides formed by the above processes can be further degraded to free amino acids by cytosolic peptidases (Figure 1*A*, Step 8).

In the five years since our last review on the ubiquitin system in this series (2), there has been an exponential increase of information on the subject. The reader is referred to reviews on the 20S and 26S proteasomes, including their subunit composition and crystal structure of the 20S proteasome from the *Thermoplasma* archaebacterium (4, 5). Ubiquitin-C-terminal hydrolases and isopeptidases are described elsewhere (6, 7). Hochstrasser's review (7) also provides a catalog of the known components of the ubiquitin system in the yeast *Saccharomyces cerevisiae*. This review discusses these subjects only briefly, focusing instead on selected examples that illustrate the mode of action and basic functions of the ubiquitin system.

ENZYMES OF UBIQUITIN-PROTEIN LIGATION

Ubiquitin Carrier Proteins (E2s)

A large number of E2s (also called Ubiquitin-conjugating enzymes or Ubcs) have been identified. In the relatively small genome of *S. cerevisiae* 13 genes

encode E2-like proteins (7), so more are likely to be found in higher eukaryotes. Some E2s have overlapping functions, whereas others have more specific roles. For example, in *S. cerevisiae*, Ubc2/Rad6 is required for DNA repair and proteolysis of so-called N-end rule substrates, Ubc3/Cdc34 is required for the G1 to S-phase transition in the cell cycle, and Ubc4 and Ubc5 are needed for the degradation of many abnormal and short-lived normal proteins (reviewed in 7, 8). Specific functions of some E2s in higher organisms have been reported. For example, *Drosophila* UbcD1[1] is needed for proper detachment of telomeres in mitosis and meiosis (9). Some mutant alleles of UbcD1 cause abnormal attachment between telomeres of sister chromatids or fusion of chromosomes through their telomere ends. UbcD1-dependent degradation of some telomere-associated proteins may be required for telomere detachment (9). Another interesting example of a specific lesion caused by a mutation in an E2 enzyme is that of the *Drosophila bendless* gene, which is required for the establishment of synaptic connectivity in development (10; see below). The inactivation of HRB6B, one of the two mouse homologs of the yeast Ubc2/Rad6 E2 enzyme, causes male sterility due to decreased spermatogenesis (11; see below). Disruption of the gene of UbcM4, a mouse E2 homologous to yeast Ubc4/Ubc5, causes embryonic lethality possibly owing to impairment of the placenta's development (12).

Because of the specific effects of mutations in some E2 genes, it was proposed that E2s may participate in the recognition of the protein substrate, either directly or in combination with an E3 enzyme (7, 8). However, not much experimental evidence exists for the direct binding of E2s to protein substrates, with the notable exception of the interactions of E2-like Ubc9 with many proteins (see below) and that of E2-25 kDa with Huntingtin, the product of the gene affected in Huntington's disease (13). Specific functions of some E2s may be the result of their association with specific E3s, which in turn bind their specific protein substrates. For example, E2-14 kDa and its yeast homolog Ubc2/Rad6 specifically bind to E3α (14) or to its yeast counterpart Ubr1p (15). Ubc2/Rad6 also binds strongly to Rad18, a yeast DNA-binding protein involved in DNA repair (16). The biochemical function of Rad18 is not known, but it may be part of an E3 complex that directs it to the site of DNA repair. The Ubc3/Cdc34 E2 protein in the budding yeast specifically associates with Cdc53p and Cdc4p, which are involved in the degradation of cell-cycle regulators necessary for the G1 to-S-phase transition (17, 18; see below).

Another specific E2 involved in cell-cycle regulation is E2-C, which was first observed as a novel E2 required for the ubiquitinylation of cyclin B in a

[1]According to the currently used nomenclature, the different E2s/Ubcs are numbered according to the chronological order of their discovery in each organism.

reconstituted system from clam oocytes (19). E2-C acts in concert with the cyclosome/APC, a large complex that has cell-cycle–regulated ubiquitin ligase activity specific for mitotic cyclins and some other cell-cycle regulators that contain the so-called destruction box degradation signal (20; see next section). E2-C from clam has a 30-amino-acid N-terminal extension and several unique internal sequences (21). Homologs of E2-C were found in *Xenopus* (22), human (23), and fission yeast (24). Expression of a dominant-negative derivative of human E2-C arrests cells in mitosis (23), as is the case with a temperature-sensitive mutant of the fission yeast homolog (24), suggesting the conservation of its cell-cycle function in evolution. However, there is no homolog of E2-C in the budding yeast, even though the subunits of the cyclosome/APC are strongly conserved in this organism (see below). The budding yeast cyclosome may act with a nonspecific E2. In a cell-free system from *Xenopus* eggs (but not in that from clam oocytes), E2-C can be replaced by the nonspecific E2 Ubc4 (25). Though the interaction of the cyclosome with E2-C has not been defined, this interaction may be less stringent in some species than in others.

Stringency of E2-E3 interactions depends not only on species but also, or mainly, on the identity of the E2 and E3 enzymes. Some E2s (for example, Ubc4) can act with more than one E3 enzyme, and some E3s can act with several E2s. For example, the ubiquitinylation of proteins by the E6-AP E3 enzyme (see next section) can be supported by UbcH5, a human homolog of yeast Ubc4 (26, 27), as well as by the closely related UbcH5B and UbcH5C (28) and the less related UbcH7 (29) (previously described as E2-F1; see 30) or UbcH8 (31). By using a yeast two-hybrid assay, researchers were able to detect interaction of E6-AP with UbcH7 and UbcH8 but not with UbcH5 (31). In contrast, UbcH5B interacts with E6-AP in an in vitro binding assay (32). These E2s may bind to E6-AP with different affinities, in which case, the strength of the binding would determine whether the association could be detected by a certain assay.

A mysterious case of an E2-like protein, Ubc9, has been solved recently. Ubc9 was originally described as an essential yeast protein required for cell-cycle progression at the G2- or early M-phase and for the degradation of B-type cyclins (33). It was proposed that the proteolytic pathway that degrades B-type cyclins involves Ubc9 (33); however, subsequent work showed that the conjugation of cyclin B to ubiquitin in a cell-free system from *Xenopus* eggs could not be supported by a *Xenopus* homolog of Ubc9 (25). Furthermore, no formation of thiolester of ubiquitin with Ubc9 could be observed following incubation with E1 and ATP (T Hadari & A Hershko, unpublished results), and the crystal structure of mammalian Ubc9 showed significant differences in the region of the active site as compared to other E2s (34).

Still, Ubc9 has important functions, as indicated by its strong conservation in many eukaryotes (see 34 and references therein). Ubc9 was identified as an

interacting protein in yeast two-hybrid searches with a surprisingly large number of proteins, including Rad51 (35) and Rad52 (36) human recombination proteins, a negative regulatory domain of the Wilms' tumor suppressor gene product (37), subunits of the CBF-3 DNA-binding complex of the yeast centromere (38), papillomavirus E1 replication protein (39), adenovirus-transforming E1A protein (40), poly (ADP-ribose) polymerase (41), transcription regulatory E2A proteins (42), the Fas (CD95) receptor of the tumor necrosis family (43, 44), and the RanBP2/RanGAP1 complex of proteins required for the action of Ran GTPase in nuclear transport (45). This last observation provided a clue to the function of Ubc9, owing to the recent discovery of the covalent modification of RanGAP1 with a small ubiquitin-like protein (46, 47). This protein has been termed UBL1 (36), sentrin (48), and SUMO-1 (47). We use the term UBL1.

The covalent ligation of UBL1 to RanGAP1 is required for its association with RanBP2, which appears to be important for the localization of the GTPase activator at the nuclear pore complex (46, 47). It was observed that a thiolester is formed between UBL1 and Ubc9, following incubation with a crude extract and ATP (M Dasso, personal communication). The reaction is analogous to the charging of E2s with activated ubiquitin and presumably involves an E1-like UBL1-activating enzyme provided by the extract. It thus appears that Ubc9 is an E2-like enzyme specific for the ligation of UBL1 to proteins. Since nuclear transport is essential for cell-cycle progression and for the degradation of mitotic cyclins (49), it was suggested that Ubc9 affects cyclin degradation indirectly, by modifying the function of RanGAP1 by ligation to UBL1 (45).

The discovery of the function of Ubc9 illustrates the importance of combining biochemical work with molecular genetic studies. It remains to be seen which other proteins are modified by ligation to UBL1 and whether at least some of the many proteins that interact with Ubc9 are also substrates for ligation to UBL1. In this system, Ubc9 may bind directly to the proteins ligated to UBL1; however, since most of the interactions of Ubc9 with various proteins were not studied with purified preparations, some of these interactions may be mediated by other proteins such as E3-like enzymes. Nonspecific interactions of some proteins with a positively charged surface of Ubc9 (34) are also possible. In vitro studies on the ligation of UBL1 to specific proteins, using purified proteins and enzymes, should resolve these questions.

Ubiquitin-Protein Ligases (E3s)

Though ubiquitin-protein ligases have centrally important roles in determining the selectivity of ubiquitin-mediated protein degradation, our knowledge of these enzymes remains limited. The difficulty in identifying new E3 enzymes is due, in part, to the lack of sequence homologies between different types of E3s, except for sequence similarities between members of the same E3 family.

In addition, some E3s are associated with large multisubunit complexes, and it is unclear which subunits of these complexes are responsible for their ubiquitin-protein ligase activities.

There is even some confusion in the literature about the properties that define an E3 enzyme. This confusion has resulted from the variety of mechanisms by which different types of E3s promote ubiquitin-protein ligation. In some cases, the protein substrate is bound directly to an E3, while in others the substrate is bound to the ligase via an adaptor molecule (see below). The mechanisms of the transfer of activated ubiquitin from a thiolester intermediate to the amino group of a protein appear to differ in various types of E3s. In some cases, E3 accepts the activated ubiquitin from an E2 and binds it as a thiolester intermediate prior to transfer to protein, while in others a ligase may help to transfer ubiquitin directly from E2 to a protein, by tight binding of E2 and the protein substrate (Figure 1*B*). The first E3 discovered, E3α, was originally defined operationally as a third enzyme component required, in addition to E1 and E2, for the ligation of ubiquitin to some specific proteins (50). We can now replace this operational definition by a more mechanistic but broad definition. We define E3 as an enzyme that binds, directly or indirectly, specific protein substrates and promotes the transfer of ubiquitin, directly or indirectly, from a thiolester intermediate to amide linkages with proteins or polyubiquitin chains.

According to this definition, four types of ubiquitin-protein ligases are known (Figure 2). The main N-end rule E3, E3α (and its yeast counterpart, Ubr1p), is still among the best-characterized ubiquitin ligases (reviewed in 2). It is an approximately 200-kDa protein that binds N-end rule protein substrates that have basic (Type I) or bulky-hydrophobic (Type II) N-terminal amino acid residues to separate binding sites specific for such residues (Figure 2*A*). Some protein substrates that do not have N-end rule N-terminal amino acid residues, such as unfolded proteins and some N-α-acetylated proteins (51), bind to this enzyme at a putative "body" site that has not been well characterized. E3α also binds a specific E2 [E2-14 kDa (14) or its yeast homolog, Ubc2p/Rad6 (15)], thus facilitating the transfer of activated ubiquitin from E2 to the substrate protein. Thus E3α is responsible for the recognition of some N-end rule protein substrates for ubiquitin ligation and degradation. A related enzyme appears to be E3β, which has been only partially purified and characterized, and which may be specific for proteins with small and uncharged N-terminal amino acid residues (52). Though the N-end rule recognition mechanism is strongly conserved in eukaryotic evolution, its main physiological functions and substrates are still not known (see below).

A second major family of E3 enzymes is the *hect* (homologous to E6-AP C-terminus) domain family. The first member of this family, E6-AP (E6-associated protein), was discovered as a 100-kDa cellular protein that was

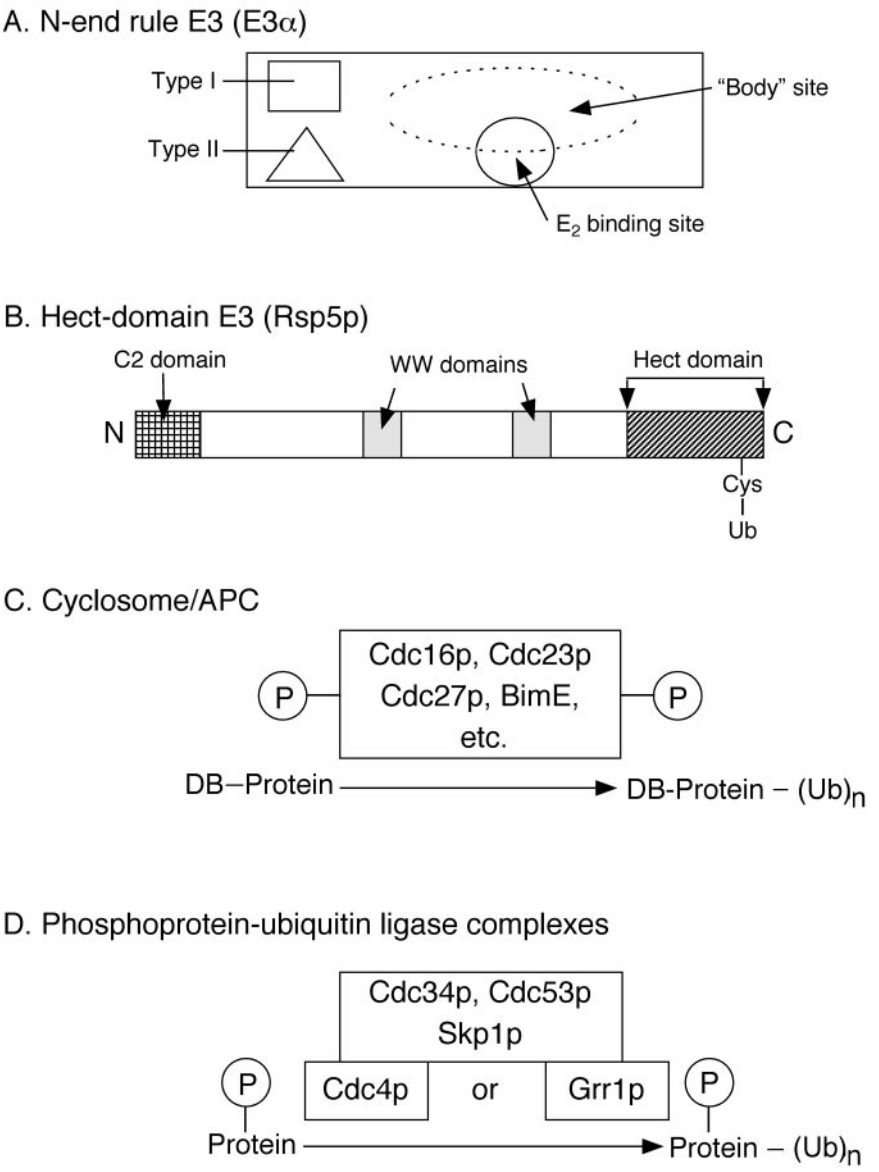

Figure 2 Different types of E3 enzymes or E3 enzyme complexes. See the text. *DB*, destruction box.

required, together with papillomavirus E6 oncoprotein, for the ubiquitinylation and degradation of p53 in reticulocyte lysates (53). In contrast to E3α, E6-AP does not bind directly to p53 but rather binds indirectly via E6, which binds to both p53 and E6-AP. In other cases, however, E6-AP can promote the transfer of ubiquitin to some cellular proteins in the absence of E6. The action of E6-AP involves an intermediary ubiquitin transfer reaction, in which activated ubiquitin is transferred from an appropriate E2 to form a thiolester with a specific Cys residue near its N-terminus (54). This thiolester is apparently the donor of ubiquitin for amide linkage with the protein substrate, because mutation of this Cys residue of E6-AP abolishes its activity in protein ubiquitinylation.

A large family of proteins that contain an approximately 350-amino-acid C-terminal region homologous to that of E6-AP, the *hect*-domain family, has been identified in many eukaryotic organisms (55; see Figure 2*B*). All *hect* proteins contain a conserved active site Cys residue near the C-terminus. In contrast to the conservation of the C-terminal domain, the N-terminal regions of the different *hect* proteins are highly variable. The N-terminal domains may be involved in the recognition of specific protein substrates (55); this has been proven in some cases (see below). Most *hect*-domain proteins are likely E3 enzymes or parts of multiprotein complexes that contain E3-like activities. At present, only fragmentary information exists about possible functions of some *hect* proteins. Some cases of Angelman syndrome, a human hereditary disease characterized by mental retardation and seizures, are due to mutations in the E6-AP gene (56, 57). This observation suggests that E6-AP-mediated protein ubiquitinylation is required for brain development (see below). More specific functions were identified for Rsp5p, one of the five *hect* proteins of the yeast *S. cerevisiae*. Rsp5p specifically binds and ubiquitinylates in vitro several yeast cellular proteins, including the large subunit of RNA polymerase II (58). The N-terminal domain of Rsp5p binds the polymerase subunit while the C-terminal (*hect*) domain does not bind, suggesting the role of the N-terminal domain in substrate binding. The relevance of these in vitro findings to similar processes occurring in vivo was suggested by the finding that inhibition of the expression of Rsp5p caused a fivefold increase in the steady-state levels of the RNA polymerase subunit (58). This subunit is usually a long-lived protein, so it is possible that it is degraded rapidly only under special conditions. In mammalian cells, the large subunit of RNA polymerase is ubiquitinylated following DNA damage induced by UV irradiation or cisplatin treatment (59) and is degraded by a proteasome-mediated process (DB Bregman, personal communication).

Two interesting problems are (*a*) How does DNA damage expose the RNA polymerase subunit to the action of the ubiquitin ligase? and (*b*) Does this process plays a role in DNA repair? Pub1, a close homolog of Rsp5p found

in fission yeast, is involved in the degradation of a different protein, the Cdc25 phosphatase (60). This phosphatase activates protein kinase Cdk1 by the removal of an inhibitory phosphate group from a tyrosine residue and thus plays an essential role in the entry of cells into mitosis. The levels of Cdc25 oscillate in the cell cycle (20). The degradation of Cdc25 is apparently mediated by the Pub1 ubiquitin ligase, as indicated by observations that disruption of *pub1* markedly increases Cdc25 levels, and *pub1* interacts with genes that control Cdc25 function. In addition, by using a mutant defective in a subunit of the 26S proteasome, researchers showed that ubiquitin conjugates of Cdc25 accumulate in pub+ but not in *pub1*-deleted cells (60).

Although in the above cases ubiquitin ligation by Rsp5p/Pub1 is apparently followed by proteasome-mediated degradation, in other instances ubiquitin ligation by the same E3 protein is involved in endocytosis (see below). Thus the general amino acid permease of the budding yeast, GAP1p, is rapidly inactivated and degraded by the addition of NH_4^+ ions. The *NPI1* gene, which is required for this process, is similar to that of the Rsp5 ubiquitin ligase (61). Rsp5p is also required for the degradation (61) and ubiquitinylation (62) of the Fur4p uracil permease. The degradation of these permeases is the result of endocytosis into the vacuole, as indicated by the finding that their degradation was inhibited in mutants of vacuolar proteases but not in mutants of proteasome subunits (see below). The action of Rsp5p on these membrane proteins may be mediated by a calcium-lipid-binding domain (CaLB/C2), which is located near the N-terminus of Rsp5p and of its homologs from other organisms (see 61 and references therein).

Another motif found in the N-terminal region of Rsp5p and of its homologs is the WW domain, an approximately 30-amino-acid region thought to be involved in interactions with proline-rich sequences containing an XPPXY (or PY) motif (see 63 and references therein). Several WW domains exist in yeast Rsp5p (see Figure 2*B*) and in Nedd4, its mammalian homolog. The rat Nedd4 was isolated as a protein that interacts with subunits of an epithelial sodium channel (63). The C-terminal tails of these channel subunits contain PY motifs. Deletion of these C-terminal tails in a human hereditary disease called Liddle's syndrome causes hypertension owing to hyperactivation of the sodium channel. Using yeast two-hybrid and in vitro binding assays, researchers showed that Nedd4 binds through its WW domains to the PY motifs of the sodium channel's subunits. It was suggested (63) and subsequently demonstrated (64) that Nedd4 suppresses the epithelial sodium channel by its ubiquitin-mediated degradation. Though Rsp5p and its homologs can directly bind at least some protein substrates, it is unknown whether they act in a monomeric form or in multiprotein complexes. In yeast, Rsp5p is associated with a protein designated Bul1, which

is not a substrate for degradation (65). A part of Rsp5p molecules is associated with Bul1 in a high-molecular-weight complex, and Bul1 may be a modulator of the Rsp5p ubiquitin ligase (65).

A high-molecular-weight complex, called the cyclosome (66) or anaphase promoting complex (APC) (25), has a ubiquitin ligase activity specific for cell-cycle regulatory proteins that contain a nine-amino-acid degenerate motif called the destruction box (Figure 2*C*; see also below). Its substrates are mitotic cyclins, some anaphase inhibitors, and spindle-associated proteins, all of which are degraded at the end of mitosis (see below). The cyclosome/APC was discovered by biochemical studies in early embryonic cell-free systems that reproduce cell-cycle-related processes. Fractionation of extracts of clam oocytes first showed that the system that ligates cyclin B to ubiquitin contained a particle-associated E3-like activity that was cell-cycle regulated. This complex was inactive in the interphase but became active at the end of mitosis, when cyclin B was degraded (19). It was dissociated from particles by extraction with high salt and was found to be an approximately 1,500-kDa complex containing destruction box–specific cyclin-ubiquitin ligase activity. The complex was named the cyclosome, to denote its large size and important roles in cell-cycle regulation (66). In the early embryonic cell cycles, the cyclosome is converted to the active form by phosphorylation (66, 67; see also below). A similar complex, called the APC, was purified from *Xenopus* eggs by immunoprecipitation (25, 68). The *Xenopus* complex has eight subunits, three of which are homologous to *S. cerevisiae* Cdc16, Cdc23, and Cdc27 proteins, which are required for exit from mitosis and for the degradation of B-type cyclins in yeasts (69). These three cyclosome subunits contain tetratrico-peptide motifs, proposed to be involved in protein-protein interactions (70). A fourth subunit is homologous to *Aspergillus nidulans* BimE protein, essential for the completion of mitosis (68, 71). These four cyclosome subunits are strongly conserved in evolution, from yeast to humans (reviewed in 72). Partial sequences obtained from four other subunits of the *Xenopus* cyclosome/APC are not homologous to proteins with known functions (68). The subunits of the cyclosome involved in its ubiquitin ligase functions, such as those responsible for specific binding to destruction box–containing substrates, and of its E2 partner, E2-C (21), have yet to be identified. Other aspects of cyclosome/APC involvement in the degradation of different cell-cycle regulators, and of the control of its activity in the cell cycle, are described in a subsequent section.

A different type of multisubunit ubiquitin ligase is involved in the degradation of some other cell-cycle regulators, such as the Sic1p Cdk inhibitor or the G1 cyclin Cln2p. In these cases, phosphorylation of the substrate converts it to a form susceptible to the action of the ubiquitin ligase complex. We designate these complexes phosphoprotein-ubiquitin ligase complexes (PULCs) (Figure 2*D*).

It appears that different PULCs exist, although present information is incomplete. These PULCs share some common components but may also have other components specific for certain protein substrates. Thus the degradation of the Cdk inhibitor Sic1p, a process essential for the G → S transition in the budding yeast, requires its phosphorylation by a G1 cyclin–activated protein kinase (73) as well as the products of *CDC34, CDC53, CDC4* (74), and *SKP1* (75). Cdc34p is an E2 protein (8), but the other gene products do not resemble proteins with known functions. Some of these components are required for the ligation of ubiquitin to Sic1p in vitro (76). Cdc34p, Cdc53p, and Cdc4p are physically associated, as indicated by their co-purification from yeast lysates (17, 18). It thus appears that a complex containing the above-mentioned components may be responsible for the ubiquitinylation of phosphorylated Sic1.

The ubiquitinylation and degradation of the yeast G1 cyclin Cln2 also requires its phosphorylation and the actions of Cdc34p, Cdc53p (17), and Skp1p (75). However, Cdc4p is not required for the degradation of G1 cyclins (S Sadis & D Finley, personal communication). Instead, the product of the *GRR1* gene is required for the degradation of the yeast G1 cyclins Cln1p and Cln2p (77). Both Cdc4p and Grr1p contain a motif called the F-box, which is present in a variety of proteins that bind to Skp1p (75). It was proposed that Skp1p is a component of ubiquitin-protein ligase complexes that connect them to specific "adaptor" proteins, such as Cdc4p and Grr1p, which would in turn bind their specific protein substrates, such as phosphorylated Sic1p and Cln2p, respectively (75). Figure 2*D* shows this model for the mode of action of different PULCs, which still has to be examined. It also remains to be seen what other specific features in protein substrates (in addition to the phosphorylated residues) are recognized by the different PULC complexes.

While the above-described information on phosphoprotein-ubiquitin ligase complexes is based on studies in yeast, it seems that at least some components of these machineries are conserved in evolution. Numerous homologs of yeast Cdc53, called cullins, were found in many eukaryotes. One of these, Cul-1, is a negative regulator of cell proliferation in *Caenorhabditis elegans* (78). A human Cdc53 homolog, Cul-2, binds to the von Hippel-Lindau tumor suppressor (79). Skp1 is also strongly conserved, and close homologs were found in many eukaryotic organisms (75). These findings suggest that similar ubiquitin ligase complexes may be involved in the degradation of a variety of regulators in higher organisms.

In addition to the four types of ubiquitin-protein ligases described above, several other E3s have been partially characterized. An approximately 550-kDa E3, designated E3L, was partially purified from rabbit reticulocytes (80). It acts on some non-N-end rule substrates, such as actin, troponin T, and MyoD. The physiological substrates of this enzyme and the signals it recognizes are

unknown. A 280-kDa E3, which ligates ubiquitin to c-fos, was purified approximately 350-fold from Fraction 2 of reticulocytes (81). An approximately 140-kDa protein was tentatively identified as a subunit of this enzyme, but the preparation was not homogenous. The formation of a thiolester between ubiquitin and the putative E3 subunit was demonstrated (81). The cloning of this E3 is necessary to examine whether it is a novel member of the *hect* family of E3 enzymes. An approximately 320-kDa E3 from reticulocytes promotes the ligation of ubiquitin to the p105 precursor of NF-κB (82).

Much remains to be learned about the identity, specificity, and regulation of E3 enzymes or E3 complexes. The lack of sequence similarity between the different types of E3 enzymes necessitates the identification of new types of E3s by biochemical methods. Because of the variety of mechanisms by which E3 enzymes carry out their two basic functions of protein substrate recognition and ubiquitin transfer, these mechanisms have to be characterized for each type of E3 enzyme. Because of these variable mechanisms, different families of E3s, specific for the recognition of different classes of protein substrates, may have evolved that do not have many features in common. The only similarity between various E3s may be the binding of E2s, but since different E3s bind different E2s, it may not be easy to recognize similarities in the various E2-binding sites. One of the major challenges in the ubiquitin field is the identification and elucidation of the mode of action of different E3s that recognize specific signals in cellular proteins.

SIGNALS IN PROTEINS FOR UBIQUITINYLATION AND DEGRADATION

Our knowledge of different signals in proteins that mark them for ubiquitinylation is also limited. Recent results indicate that many proteins are targeted for degradation by phosphorylation. It was observed previously that many rapidly degraded proteins contain PEST elements, regions enriched in Pro, Glu, Ser, and Thr residues (83, 84). More recently, it was pointed out that PEST elements are rich in S/TP sequences, which are minimum consensus phosphorylation sites for Cdks and some other protein kinases (85). Indeed, it now appears that in several (though certainly not all) instances, PEST elements contain phosphorylation sites necessary for degradation. Thus multiple phosphorylations within PEST elements are required for the ubiquitinylation and degradation of the yeast G1 cyclins Cln3 (85) and Cln2 (86), as well as the Gcn4 transcriptional activator (87). Other proteins, such as the mammalian G1 regulators cyclin E (88) and cyclin D1 (89), are targeted for ubiquitinylation by phosphorylation at specific, single sites. In the case of the IκBα inhibitor of the NF-κB transcriptional regulator, phosphorylation at two specific sites, Ser32 and Ser36, is required for ubiquitin ligation (see below). β-Catenin, which is targeted

for ubiquitin-mediated degradation by phosphorylation (see below), has a sequence motif similar to that of IκBα around these phosphorylation sites (90). However, the homology in phosphorylation patterns of these two proteins is not complete, because phosphorylation of other sites of β-catenin is also required for its degradation (90; see below).

Other proteins targeted for degradation by phosphorylation include the Cdk inhibitor Sic1p (73) and the STAT1 transcription factor (91). Though different patterns of phosphorylation target different proteins for degradation, a common feature appears to be that the initial regulatory event is carried out by a protein kinase, while the role of a ubiquitin ligase would be to recognize the phosphorylated form of the protein substrate. It further appears that different ubiquitin ligases recognize different phosphorylation patterns as well as additional motifs in the various protein substrates. However, the identity of such E3s is unknown, except for some PULC-type ubiquitin ligases that act on some phosphorylated cell-cycle regulators in the budding yeast (see previous section). The multiplicity of signals that target proteins for ubiquitin-mediated degradation (and of ligases that have to recognize such signals) is underscored by observations that the phosphorylation of some proteins actually prevents their degradation. Thus the phosphorylation of the c-Mos protooncogene on Ser3 (92) and the multiple phosphorylations of c-Fos (93) and c-Jun (94) protooncogenes at multiple sites by MAP kinases suppress their ubiquitinylation and degradation (see also below).

Among degradation signals inherent in primary protein structure, the best characterized is still the N-end rule system, in which the ubiquitinylation and degradation of a protein is determined by the nature of its N-terminal amino acid residue (reviewed in 95). However, there are few known physiological protein substrates of this system, presumably because of the specificity of methionine aminopeptidases that do not remove initiating Met residues from nascent proteins when the second amino acid residue is an N-end rule destabilizing residue (96). An important function of this pathway may be to remove from the cytosol erroneously transported or compartmentalized proteins, in which a destabilizing N-terminal residue is produced in cleavage by a signal peptidase. Because the few known physiological substrates of the N-end rule system, such as the Gα subunit of G-protein (97) or the CUP9 transcriptional repressor of peptide import in yeast (95), do not have destabilizing N-terminal residues, they are presumably recognized by some other internal signal.

A signal important for the degradation of mitotic cyclins and certain other cell-cycle regulators is the destruction box. It was first discovered as a partially conserved, 9-amino-acid sequence motif usually located approximately 40–50 amino acid residues from the N-terminus of mitotic cyclins and is necessary for their ubiquitinylation and degradation in extracts of *Xenopus* eggs (98). Compilation of destruction box sequences from nearly 40 B-type and A-type

cyclins from various organisms (99) showed that they have the following general structure:

R	(A/T)	(A)	L	(G)	x	(I/V)	(G/T)	(N)
1	2	3	4	5	6	7	8	9

Amino acid residues, or combinations of two residues, that appear in parentheses in the above structure occur in more than 50% of known destruction sequences. Thus the only invariable residues are R and L in positions 1 and 4, respectively; the rest of the destruction box sequence is quite degenerate.

Still, the destruction box signal is absolutely necessary for the ubiquitinylation and degradation of mitotic cyclins in vitro (66, 100) and in vivo (see 99 and references therein), as shown by the prevention of these processes by deletion of the destruction box region or by point mutations in its conserved residues. Moreover, the destruction box–containing N-terminal fragments of mitotic cyclins act as transferable signals in vitro and in vivo, as indicated by the cell-cycle-stage-specific degradation of reporter proteins fused to such fragments (98, 100, 101). Similar destruction box motifs are required for the degradation of certain non-cyclin cell-cycle regulators that are degraded at late mitosis, such as anaphase inhibitors and the spindle-associated protein Ase1p (see below).

All presently known destruction box–containing cell-cycle regulators are ligated to ubiquitin by the cyclosome/APC and are degraded after the conversion of the cyclosome to the active form at late mitosis. However, some destruction box–containing proteins are degraded at slightly different times during the cell cycle, indicating additional levels of regulation (see below). Thus the cyclosome-mediated ubiquitinylation of destruction box–containing proteins may be an example (thus far, unique) of a strategy by which a limited set of proteins that perform related functions and share a common degradation signal are substrates for a common ubiquitin ligase. Some proteins that are not related to cell-cycle regulation, such as the budding yeast Ras exchange factor Cdc25p (102) and uracil permease (61), have been reported to be degraded in a destruction box–related manner, but it is unknown whether cyclosome action is involved in these cases.

Much less is known about signals or domains recognized for degradation in other cellular proteins. Truncations or deletions of several rapidly degraded proteins cause their stabilization (e.g., see 103, 104), but since it has not been shown that these regions contain transferable degradation signals, stabilization may be the result of secondary effects on protein structure. An exception is the case of c-Jun, in which the δ-domain of a sequence of 27 amino acid residues near the N-terminus is a transferable ubiquitinylation signal (105; see also below).

DEGRADATION OF UBIQUITIN-PROTEIN CONJUGATES

The 20S and 26S Proteasome Complexes

The structure and function of the 20S and 26S proteasome complexes have been reviewed elsewhere (see 4, 6, 7, 106–109). In this section, we update the reader on some important recent developments.

Important progress has been made in the resolution at 2.4 Å of the crystal structure of the eukaryotic (yeast) 20S proteasome (110). This study corroborated previous observations on the structure of the complex from the archeabacterium *Thermoplasma acidophilum* but also revealed some unexpected features. Like the *T. acidophilum* proteasome, the yeast complex is also arranged as a stack of four rings, each containing seven subunits, $\alpha_7\beta_7\beta_7\alpha_7$. The catalytic sites reside in some of the β rings. However, the composition of the eukaryotic 20S proteasome is more complicated than that of the archaeal complex. While each of the *T. acidophilum* proteasome rings is composed of identical subunits, seven identical α subunits for each of the two α rings and seven identical β subunits for each of the two β rings, the rings of the yeast enzyme are composed of seven distinct subunits. Thus, the 20S proteasome of yeast is composed of 14 pairs of protein subunits, 7 different α and 7 different β subunits organized as $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$.

Resolution of the crystal structure enabled better understanding of the biogenesis of the different chains. Five β-type subunits are synthesized as proproteins with N-terminal extensions of up to 75 residues and are cleaved during proteasome maturation (reviewed in 4, 110). Three of the subunits (β1/PRE3, β2/PUP1, and β5/PRE2) undergo cleavage between the last Gly residue of the pro-peptide and Thr^1 of the mature subunit that also constitutes the catalytic site. The enzymes use the side chain of the Thr residue as a nucleophile in a catalytic attack at the carbonyl carbon. Activation of the side chain occurs by transfer of its proton to the free N-terminus. The Thr residue occupies an unusual fold (common also to other aminohydrolases such as glutamine PRPP amidotransferase, the penicillin acylase, and the aspartylglucosaminidase), which provides the capacity for both the nucleophilic attack and autocatalytic processing.

Several other adjacent preserved residues in β-type subunits (Gly^{-1}, Asp^{17}, Lys^{33}, Ser^{129}, Asp^{166}, and Ser^{169}) are also important for the structural integrity of the catalytic site (111–113). Topological analysis of the location of the different subunits has revealed that for the three distinct proteolytic activities—the trypsin-like, the chymotrypsin-like, and the post-glutamyl peptidyl hydrolytic (PGPH) activities—the active sites are generated by adjacent pairs of identical β-type subunits residing in different β rings. These findings have been corroborated independently by genetic analysis (114), as well as by

immunoelectronmicroscopy and chemical cross-linking of neighboring subunits (115). The crystal structure has also shown that the α chains, although catalytically inactive, play an essential role in stabilizing the two-ring structure of the β chains. They must also play a role in the binding of the 19S cap or regulatory complexes, but the structure of the contacts and mechanisms of binding will be elucidated only when the structure of the 26S complex is resolved. The crystal structure has revealed a distance of 28 Å between the Thr^1 active sites of adjacent active β subunits. This distance may determine the length of the peptides generated during the proteolytic process ($\sim$8 amino acid residues) and may explain the role of the proteasome in generation of antigenic peptides presented on class I MHC molecules (109, 116, 117; see also below).

An unresolved problem involves the entry of protein substrates into, and exit of proteolysis products from, the proteasome. In the *T. acidophilum* proteasome, there are two putative entry pores of approximately 13 Å at the two ends of the cylinder surrounded by defined segments of the seven α subunits (118). In striking and rather surprising contrast, these pores do not exist in the yeast 20S proteasome, and entry to the inter-β rings catalytic chamber is not possible from the ends of the complex. The N-terminal domains of α1/C7, α2/Y7, α3/Y13, α6/PRE5, and α7/C1 protrude toward each other and fill the space in several layers of tightly interacting side chains (110). Thus, entry from the ends may be possible only after substantial rearrangement that can occur after association with the 19S regulatory complex. Such a rearrangement may also require energy that can be provided by the ATPase activity of the 19S regulatory complex. Also, unlike the *T. acidophilum* proteasome, the yeast complex displays some narrow side orifices, particularly at the interface between the α and β rings. These openings lead directly to the Thr^1 active sites. They are coated with polar residues that can potentially rearrange to generate $\sim$10-Å apertures through which unfolded and extended protein substrates may enter.

Substrate recognition by the 26S proteasome is probably mediated by the interaction of specific subunits of the 19S regulatory complex with polyubiquitin chains. Indeed, such subunits have been described both in humans (S5a; see 119) and in plants (MBP1; see 120). These subunits bind at high-affinity polyubiquitin chains, in particular those that contain more than four moieties, but they also bind ubiquitin markers. The association of these subunits with the 19S complex and their preference for polyubiquitinylated tagged substrates suggests a crucial role for these subunits in ubiquitin-mediated protein degradation. *Mcb1*, the yeast gene encoding the homologous subunit was cloned recently. Surprisingly, Δ*mcb1* deletion mutants do not display any growth defect and degrade normally ubiquitinylated proteins, except for the fusion model protein ubiquitin-Pro-β-Gal. These mutants do display a slight sensitivity to

stress, such as exposure to amino acid analogs (121). A possible explanation for these results is that ubiquitinylated proteins are recognized by additional, as-yet-undefined proteasomal subunits.

Specific inhibitors of the proteasome have proved to be important research tools, probing the structure and function of the proteasome and establishing the involvement of the ubiquitin-proteasome pathway in the degradation of specific proteins. The initial 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 most cellular proteins, both short and long lived (122). They modify covalently and irreversibly the Thr^1 in the catalytically active β subunits. While they are quite specific toward the proteasome, at higher concentrations, they also inhibit calpains. By contrast, the *Streptomyces* metabolite lactacystin appears to be a specific inhibitor of the proteasome (123). It modifies covalently the active site Thr^1 residues and strongly inhibits the trypsin- and chymotrypsin-like activities of the complex and, less efficiently, the PGPH activity. A recently developed derivative of the calpain inhibitors, carboxybenzyl-Leu-Leu-Leu-vinyl sulfone (Z-L_3VS) inhibits efficiently and specifically all three activities of the proteasome (124). It is cell permeable and inhibits the activity of the complex in vivo as well. Although vinyl sulfone derivatives were described originally as cysteine protease inhibitors, like all other known inhibitors of the proteasome, these derivatives covalently modify the Thr^1 residues in active β subunits.

Ubiquitin-C-Terminal Hydrolases and Isopeptidases

The subject of ubiquitin-C-terminal hydrolases (UCHs) and ubiquitin-specific proteases (UBPs) (also called isopeptidases and de-ubiquitinating enzymes) is reviewed elsewhere (6, 7, 125), and we discuss here only some recent developments. Genes of 16 different UBPs are found in the yeast genome (7). The large number of hydrolases suggests that some of them may have specific functions, such as the recognition of different types of ubiquitin conjugates. Thus a family of low-molecular-mass (25- to 28-kDa) UCHs specifically act on adducts of ubiquitin with small molecules or peptides (126). The crystal structure of one of these, UCH-L3, has been solved at 1.8 Å resolution (127). The enzyme comprises a central antiparallel β-sheet flanked on both sides by α helices. The β-sheet and one of the helices are similar to those observed in the thiol protease cathepsin B. The similarity includes the three amino acid residues that comprise the active site, Cys^{95}, His^{169}, and Asp^{184}. The active site appears to fit the binding of ubiquitin that may anchor also at an additional site. The catalytic site in the free enzyme is masked by two different segments of the molecule that limit nonspecific hydrolysis and must undergo conformational rearrangement after substrate binding.

Another hydrolase, isopeptidase T (IsoT), acts preferentially on free, unanchored polyubiquitin chains and stimulates protein breakdown by the disassembly of such chains that inhibit the action of the 26S proteasome (128). IsoT acts by a sequential *exo* mechanism, starting from the end of the polyubiquitin chain that contains a free C-terminus of ubiquitin (125). This free C-terminus can be exposed following the action of the 26S proteasome on the protein moiety of polyubiquitin-protein conjugates. A recent report (129) describes the characterization of Ubp14, the yeast homolog of IsoT. Like IsoT, Ubp14 is involved in disassembly of free, unanchored polyubiquitin chains. A Δ*Ubp14* mutant, as well as a yeast expressing a dominant-negative mutant form of the enzyme, display a lowered rate of general protein degradation accompanied by accumulation of free ubiquitin chains, probably bound to the proteasome. Unexpectedly, overexpression of the wild-type protein also results in inhibition of proteolysis of certain proteins. It is possible that certain substrates are tagged by direct transfer of polyubiquitin chains, and the low level of such chains resulting from overexpression of the wild-type enzyme leads to inhibition of their degradation. Complementation experiments have revealed that Ubp14 and IsoT are functional homologs, confirming in vivo the initial characterization of the enzyme carried out in a cell-free system using a model substrate.

The action of the UCHs and IsoT stimulates protein breakdown by the removal of inhibitory polyubiquitin chains and by the regeneration of free and reusable ubiquitin. In other cases, the action of an isopeptidase may inhibit protein breakdown. For example, a mutation in the *Drosophila faf facets* (*faf*) gene, which encodes an isopeptidase affecting eye development (see below) is suppressed by another mutation in a proteasome subunit (130). These results indicate that the *faf* isopeptidase stabilizes some unidentified proteins, which are also stabilized by the proteasome mutation. It is possible that certain isopeptidases can stabilize particular proteins by the removal of ubiquitin from conjugates that would be otherwise targeted for degradation by the 26S proteasome. An editing function for some isopeptidases was proposed a long time ago (131). Recently, Lam et al (132) reported that the 19S regulatory complex of the 26S proteasome contains a 37-kDa ubiquitin-aldehyde-sensitive but ATP-independent isopeptidase that removes single ubiquitin moieties from the distal end of short polyubiquitin chains. The authors proposed that this isopeptidase is involved in editing and in rescue of poorly ubiquitinated or slowly degraded proteins from degradation, which differs from the function of isopeptidase Doa4 (135) and the ATP-dependent but Ubal-insensitive isopeptidase (136) involved mostly in recycling of ubiquitin and maintenance of free ubiquitin levels in the cell.

Low concentrations of ubiquitin aldehyde, an inhibitor of some isopeptidases (133) stimulates the degradation of excess globin α-chains in reticulocytes of

thalassemic patients (134). This observation suggests that ubiquitin conjugates of α-globin are disassembled by an isopeptidase that prevents its degradation by the 26S proteasome.

Different ubiquitin-C-terminal isopeptidases affect a variety of other basic processes, including development (see below), gene silencing (137), and long-term memory (138). In none of these cases were the target proteins identified, nor was the mode of action of the isopeptidase characterized in the degradation or stabilization of target proteins. Another interesting function of specific isopeptidases is the regulation of cell proliferation. It was observed that cytokines induced in T-cells specific de-ubiquitinating enzymes (DUBs), termed DUB-1 (139) and DUB-2 (140). DUB-1 is induced by stimulation of the cytokine receptors for IL-3, IL-5, and GM-CSF, suggesting a role in its induction for the β-common (betac) subunit of the interleukin receptors. Overexpression of a dominant negative mutant of JAK2 inhibits cytokine induction of DUB-1 (141), suggesting that the regulation of the enzyme is part of the cell response to the JAK/STAT signal transduction pathway. Continued expression of DUB-1 arrests cells at G_1; therefore, the enzyme appears to regulate cellular growth via control of the G_0-G_1 transition. The catalytic conserved Cys residue of the enzyme is required for its activity. DUB-2 is induced by IL-2 as an immediate early (IE) gene that is down-regulated shortly after the initiation of stimulation. The function of this enzyme is also obscure. It may stimulate or inhibit the degradation of a critical cell-cycle regulator (see below).

CELLULAR PROTEINS DEGRADED BY THE UBIQUITIN SYSTEM

Cell-Cycle Regulators

Progress in the eukaryotic cell-cycle is driven by oscillations in the activities of cyclin-dependent kinases (Cdks). Cdk activity is controlled by periodic synthesis and degradation of positive regulatory subunits, cyclins, as well as by fluctuations in levels of negative regulators, by Cdk inhibitors (Ckis), and by reversible phosphorylation (reviewed in 142). The different cyclins, specific for the G1, S-, or M-phases of the cell cycle, accumulate and activate Cdks at the appropriate times during the cell cycle and then are degraded, causing kinase inactivation. Levels of some Ckis, which specifically inhibit certain cyclin/Cdk complexes, also rise and fall at specific times during the cell cycle. Selective, ubiquitin-mediated degradation of cyclins, Ckis, and other cell-cycle regulators appear to play centrally important roles in cell-cycle control, as described below.

MITOTIC CYCLINS Though all cyclins are degraded by ubiquitin-mediated processes, the systems that carry out their ligation to ubiquitin, and the mode by

which these systems are connected to the cell-cycle regulatory phosphorylation network, are different for mitotic and G1 cyclins. Mitotic B-type cyclins and some S-phase cyclins such as cyclin A (66) are ligated to ubiquitin by the cyclosome, while G1 cyclins are ubiquitinylated by PULC-type E3 enzymes (see previous section). In the former case, the activity of the ligase is regulated in the cell cycle, whereas the latter process is triggered by the phosphorylation of the G1 cyclin substrate. The mitotic cyclin B was the first cyclin discovered, by its striking degradation at the end of each mitosis in early sea urchin embryos (143). Cyclin B combines with Cdk1 (also called Cdc2 or Cdc28 in the fission or budding yeasts, respectively) to form the major mitotic kinase MPF (M-phase promoting factor). MPF causes entry of cells into mitosis and, after a lag, activates the system that degrades its cyclin subunit (reviewed in 20). MPF inactivation, caused by the degradation of cyclin B, is required for exit from mitosis, as shown by observations that cells expressing nondegradable forms of cyclin B arrest in late anaphase (144).

Initial evidence indicating that the degradation of cyclin B is carried out by the ubiquitin system was based on correlations between the degradation of cyclin B and its ubiquitinylation in extracts of *Xenopus* eggs (98), and on the inhibition of cyclin degradation by methylated ubiquitin (an inhibitor of polyubiquitin chain formation; see 145) in extracts of clam oocytes (146). Fractionation of these extracts (19) led to the identification of the specific components of this system, the novel ubiquitin-carrier protein E2-C (21), and the cyclosome complex that has cyclin-ubiquitin ligase activity (66; see also previous section). E2-C is constitutively active, but the activity of the cyclosome is cell-cycle regulated: It is inactive in the interphase of the embryonic cell cycle and is converted to the active form at the end of mitosis by phosphorylation (19, 25, 66; see also below). Thus the regulation of the degradation of mitotic cyclins in the cell cycle is carried out mainly by the modulation of the cyclin-ubiquitin ligase (E3) activity of the cyclosome (67).

Molecular genetic studies in intact cells, mainly in yeasts, corroborated the results of biochemical studies on the mode of ubiquitin-mediated degradation of mitotic cyclins. The degradation of B-type cyclins in yeasts requires functional subunits of the 26S proteasome (147, 148). Most significantly, the discovery that products of the budding yeast genes CDC16 and CDC23 are required for cyclin B proteolysis in vivo (69) led to the identification of their homologs as subunits of the cyclosome/APC (68). Other cyclosome subunits, such as homologs of BimE of *A. nidulans*, are also required for the degradation of B-type cyclins in both the budding (71) and fission (149) yeasts.

The molecular mechanisms regulating the machinery that degrades cyclin B are not well understood. In the relatively simple early embryonic cell cycles, it seems that the activity of the cyclosome is mainly regulated by its reversible

phosphorylation, as indicated by the observations that the inactive, interphase form of the cyclosome can be converted in vitro to the active form by incubation with MPF (19, 66) and that the active, mitotic form of the cyclosome can be converted to the inactive form by treatment with an okadaic acid–sensitive phosphatase (67). Conversion of the cyclosome to the active form by MPF, previously observed with partially purified preparations, has been confirmed using highly purified preparations of cyclosome from clam oocytes (M Shteinberg & A Hershko, unpublished results), indicating that activation is due to direct phosphorylation of the cyclosome by MPF.

Cyclosome activation by MPF-dependent phosphorylation may involve the action of the suc1/cks family of proteins. These proteins were discovered in yeasts as gene products that interact with Cdk1 and were subsequently found in higher organisms (reviewed in 150). In yeasts, suc1/cks proteins are required at several stages of the cell cycle, including entry into mitosis, exit from mitosis, and the degradation of B-type cyclins. Immunodepletion experiments in extracts of *Xenopus* eggs also indicated that suc1/cks has multiple roles in the cell cycle, including the degradation of cyclin B (151). The requirement of cyclin degradation for suc1/cks may be explained by the recent finding that the active, phosphorylated form of the cyclosome binds to $p13^{suc1}$ beads (152). Several lines of evidence indicated that the cyclosome does not bind to the Cdk-binding site but rather to a phosphate-binding site of suc1. Thus the cyclosome could be eluted from suc1-Sepharose beads by phosphate-containing compounds, an observation used to develop a procedure for the affinity purification of the cyclosome (152).

A conserved phosphate-binding site was found by x-ray crystallography (in addition to the Cdk-binding site) in all suc1/cks proteins, and researchers suggested that this site directs Cdks to some phosphorylated proteins (150). If multiple phosphorylations are required for cyclosome activation, initial slow phosphorylations may cause tighter binding of MPF to the cyclosome via suc1/cks, thus accelerating additional phosphorylations. Such a model may explain, at least in part, the lag kinetics of interphase cyclosome activation by MPF. This lag, which can be reproduced in vitro, presumably plays an important role in preventing premature self-inactivation of MPF prior to the end of mitosis. This model of the possible role of suc1/cks proteins in the kinetics of cyclosome activation remains to be investigated.

Information on the regulation of cyclin B degradation is based on studies in relatively simple early embryonic cell-cycle systems, which consist of rapidly alternating S- and M-phases, without any intervening G1 and G2 phases. The regulation of mitotic cyclin degradation is more complicated in the more complex cell cycles of somatic cells and unicellular eukaryotes, which contain many additional events in the G1 and G2 phases and have to respond to a variety of

extracellular stimuli. Thus the activation of the B-type cyclin proteolysis machinery in yeasts also occurs at the end of mitosis but requires several gene products (reviewed in 153), including a protein phosphatase (154). This process is very different from the regulation of the early embryonic cyclosome, which is inactivated by phosphatase action (67). It is unknown whether the phosphatase acts on the cyclosome directly, removing an inhibitory phosphate group, or whether it is a part of a signal transduction system that affects the cyclosome indirectly.

The inactivation of the cyclin-degrading machinery in yeast or somatic cells is also very different from that of early embryos. In early embryos the cyclin-degrading system is active for only a few minutes at the end of mitosis (155), whereas in yeast it remains active until the end of the G1 phase of the next cell cycle, when it is turned off by the action of G1 cyclins (156). It is unknown how the B-type cyclin proteolytic machinery is turned off by G1 cyclins, but some phosphorylation event by G1 cyclin/Cdk complexes likely inhibits cyclosome activity directly or indirectly. Similarly, in cultured mammalian cells the proteolysis of mitotic cyclins is activated shortly before anaphase and is turned off only at the end of the G1 phase of the next cell cycle (101). The continued activity of this degradation machinery during the G1 phase of the cell cycle may be needed to prevent the premature accumulation of S-phase cyclin substrates of the cyclosome. More research is needed on the mechanisms by which cyclosome activity is regulated in the somatic type of cell cycles.

G1 CYCLINS Cyclins specific for the G1 phase of the cell cycle are also highly unstable proteins. G1 cyclins do not contain a destruction box motif but are targeted for degradation by phosphorylation. The phosphorylation of yeast G1 cyclins Cln2p (86) and Cln3p (85) is required for their degradation. Available information indicates that phosphorylated G1 cyclins may be ligated to ubiquitin by PULC-type complexes. Thus the degradation of both Cln2p (17) and Cln3p (85) requires the Cdc34p E2 enzyme. Ligation of Cln2 to ubiquitin and its degradation both require Cdc53p, and the phosphorylated form of Cln2p co-purifies with Cdc53p (17). The degradation of Cln2p requires the action of *SKP1* (75), and the degradation of Cln1p and Cln2p require *GRR1* (77). Although evidence for the involvement of PULCs in the degradation of yeast G1 cyclins is much less complete than that available for the Sic1p Cdk inhibitor (see below), similar complexes likely carry out the ubiquitinylation of yeast G1 cyclins.

Available information on the mode of degradation of mammalian G1 cyclins is much more limited, but it appears that in this case, too, phosphorylation of the cyclin substrate is required for its ubiquitin-mediated degradation. However, in contrast to the multiple phosphorylations required for the degradation of yeast Cln2p (86), phosphorylation of specific, single sites has a strong influence on

the degradation of mammalian G1 cyclins. Thus the rapid degradation of human cyclin E is markedly slowed by mutation of a specific Cdk phosphorylation site at T380 (88, 157). Residue T380 of cyclin E is phosphorylated in vivo and autophosphorylated in vitro (88). When cells were treated with proteasome inhibitors, the accumulation of ubiquitinylated derivatives of wild-type cyclin E (88, 157), but not of the T480A mutant of cyclin E, was observed. It was suggested that autophosphorylation of cyclin E initiates its ubiquitinylation and degradation (88). The degradation of another mammalian G1 cyclin, cyclin D1, also requires its specific phosphorylation at T286 (89). In this case, too, proteasome inhibitors caused the accumulation of ubiquitinylated derivatives of wild-type cyclin D1, but not of the T286A mutant of cyclin D1, indicating that phosphorylation is required for ubiquitinylation (89). The ubiquitinylation systems that act on phosphorylated mammalian G1 cyclins remain to be identified.

CDK INHIBITORS The activities of some Cdks are controlled tightly by fluctuations in the levels of their negative regulatory proteins, Ckis. Thus a cyclin/Cdk complex cannot act until the inhibitor is removed by selective proteolysis. A well-studied case is that of the Sic1p Cdk inhibitor of the budding yeast, *S. cerevisiae*. Sic1p inhibits the activity of complexes of Cdk1 with B-type cyclins but does not inhibit the activity of G1 cyclin/Cdk1 complexes (74). Levels of Sic1p are high in the G1 phase of the cell cycle, but the inhibitor is degraded rapidly at the G1 to S-phase transition. The degradation of Sic1p is a centrally important event in the transition from G1 to the S-phase, because it permits the action of S-phase-promoting B-type cyclins to initiate DNA replication (74). The degradation of Sic1p requires its prior phosphorylation by G1 cyclin/Cdk1 complexes, as indicated by the findings that Sic1p is phosphorylated at multiple sites at the end of G1, and this phosphorylation depends on the activities of G1 cyclins (73).

Sic1p degradation also requires the action of the Cdc34p E2 enzyme, as well as that of the products of the *CDC4*, *CDC53* (74), and *SKP1* (75) genes. Biochemical experiments in which the ligation of Sic1p to ubiquitin was reconstituted in vitro indicated that the products of most of these genes are directly required for this process (76). Most of the components required for Sic1p ubiquitinylation have been shown to be assembled in a multiprotein complex (18). Thus phosphorylated Sic1p is likely targeted for degradation by a PULC-type complex (Figure 2*D*); however, the action of PULCs may not be specific for cell-cycle regulators. For example, the degradation of the Gcn4 transcriptional activator of amino acid biosynthesis in yeast, which takes place throughout the cell cycle, requires its phosphorylation and the action of *CDC34* (87) as well as *CDC53*, *CDC4*, and *SKP1* genes (D Kornitzer, personal communication). In contrast to the regulation of the cyclosome, the activity of which is turned on

and off at specific points of the cell cycle, PULCs may be constitutively active. The precise timing of the degradation of Sic1p is apparently determined by the accumulation of G1 cyclin/Cdk1 complexes in late G1, which phosphorylate Sic1p and thus trigger its degradation.

In the fission yeast *Schizosaccharomyces pombe*, a similar function is carried out by the rum1 protein, a specific inhibitor of cyclin B/Cdk1 complexes (158). In this case, too, levels of rum1 are high in G1, but the inhibitor is degraded upon transition to the S-phase. The mode of the degradation of rum1 is not known, except that it requires pop1, the fission yeast homolog of Cdc4p (159). A different function is carried out by another budding yeast Cdk inhibitor, Far1p: It accumulates in response to mating pheromone and arrests cells in early G1 owing to the inhibition of G1 cyclin/Cdk1 complexes (160). Thus Far1p action mediates response to extracellular signals, as is the case with some vertebrate Cdk inhibitors (see below). Upon release of yeasts from arrest in early G1, Far1p is degraded rapidly (161). The degradation of Far1p is preceded by its phosphorylation (161) and requires the function of Cdk1 (162). As a result, the phosphorylation of Far1p is likely required for its degradation. The machinery that carries out the degradation of Far1p is not known, except that Far1p accumulates in *cdc34* or *cdc4* mutants (161), and it is markedly stabilized by the deletion of its 50 N-terminal amino acids (162).

Many Ckis have been identified in mammalian cells, and these can be divided into two families based on sequence similarities: The KIP/CIP family contains p21, p27, and p57, and the INK family includes p15, p16, p18, and p19 (163). All mammalian Ckis inhibit G1 cyclin/Cdk complexes (though with different specificities) and thus mediate cell-cycle arrest in response to a variety of growth-inhibiting conditions. For example, p21 is induced by DNA damage in a process mediated by p53 (163), p27 levels are increased greatly in cells arrested by deprivation of growth factors or contact inhibition (164), and p18 levels are elevated in terminal differentiation associated with permanent cell-cycle arrest (165).

Several mammalian Ckis are unstable proteins, the levels of which may be modulated by alterations in the rates of their degradation. Thus the high levels of p27 in quiescent cells result, at least in part, from decreased degradation (166, 167). After growth stimulation, p27 levels decrease rapidly owing to degradation by the ubiquitin system, as indicated by the accumulation of its ubiquitinylated derivatives following treatment of cells with proteasome inhibitor (166). Furthermore, rates of ubiquitinylation of p27 in vitro are higher in extracts of proliferating cells than in those of quiescent cells (166). Co-overexpression of different derivatives of p27 with Cdk2/cyclin E led to the suggestion that phosphorylation of p27 by cdk2/cyclin E on T187 causes its degradation (168). Since p27 inhibits the action of cdk2/cyclin E, it was further

proposed that the phosphorylation of p27 by the kinase is faster than the formation of the inhibited complex (168). It is unclear whether the ratio of p27 to Cdk2/cyclin E in these overexpression experiments is comparable to the physiological situation. Thus, the identity of the system that ligates p27 to ubiquitin and the mode of the regulation of this process remain to be identified.

The p21 Cki is also a rapidly degraded protein that is ubiquitinylated in vivo (169). Another interesting case is that of a p15 Cki, the degradation of which is inhibited by transforming factor β, thus causing its accumulation (170). Based on these findings, the regulation of Cdk inhibitor–degradation in animal cells may be involved in connecting to the basic cell-cycle machinery a variety of signals that affect cell proliferation.

ANAPHASE INHIBITORS AND OTHER CELL-CYCLE REGULATORS In addition to cyclins and Ckis, the levels of numerous other cell-cycle regulators oscillate during the cell cycle. Some of these regulators are targeted for degradation by cyclosome-mediated ubiquitin ligation, some by PULC-type mechanisms, and for others, the mechanisms are unknown. In extracts of *Xenopus* eggs, the addition of an N-terminally truncated, nondegradable derivative of cyclin B caused arrest at late anaphase, while the addition of the N-terminal fragment of cyclin B caused an earlier arrest at the metaphase (171). This observation indicates that the machinery that degrades mitotic cyclins is also involved in the degradation of other cell-cycle regulators. Since the N-terminal fragment of cyclin B contains the destruction box region, it was suggested that the degradation of some other destruction box–containing protein (which is competed by the N-terminal fragment of cyclin B) is required for the separation of sister chromatids that takes place in the metaphase-anaphase transition (171).

These in vitro observations were confirmed in vivo (99); however, the identity of the putative anaphase inhibitors remained unknown until recently. One of these inhibitors is the Cut2 protein of *S. pombe*, which is localized on the mitotic spindle and is degraded rapidly at the end of the anaphase (172). The degradation of Cut2 requires the presence of two destruction box regions at its N-terminal domain (173) and the activity of the cyclosome (172). Most significantly, the expression of nondegradable derivatives of Cut2 prevented the separation of sister chromatids. As a result, destruction box–dependent, cyclosome-mediated degradation of Cut2 may be required for the onset of the anaphase (172). Essentially similar results were observed on Pdsp1p, another anaphase inhibitor in *S. cerevisiae* (174).

The mode of action of anaphase inhibitors is unknown. They may act as "molecular glues" that hold sister chromatids together until they are degraded, or they may act by much more indirect mechanisms. Another destruction box–containing protein in the budding yeast, Ase1p, is bound to the midzone of

the mitotic spindle and is degraded at the end of mitosis by a mechanism that requires the activity of the cyclosome (175). This process may be involved in the disassembly of the mitotic spindle, since the expression of a nondegradable derivative of Ase1 caused a delay in spindle disassembly (175). It seems that the cyclosome is responsible for the degradation of various cell-cycle regulators at the end of mitosis, all of which share the destruction box–recognition determinant.

Another important unstable cell-cycle regulator is the Cdc6 protein of the budding yeast, which is required for the initiation of DNA replication (reviewed in 153, 176). Cdc6p is synthesized in G1, associates with the origin replication complex, and promotes its conversion to a form competent for replication. After the initiation of DNA replication, Cdc6p is degraded rapidly (177). A similar function is carried out in the fission yeast by a homologous protein, Cdc18 (178). In the fission yeast, the elimination of Cdc18 after the initiation of DNA replication may prevent replication of DNA more than once in a cell cycle, since massive overexpression of Cdc18 caused repeated rounds of DNA synthesis, without mitosis (178). In contrast, ectopic expression of Cdc6p in the budding yeast after G1 does not cause re-replication (177). In the latter case, the prevention of re-replication appears to be tightly controlled by other mechanisms; thus the degradation of Cdc6p may be an additional safeguard to ensure that DNA is replicated only once in a cell cycle. The mechanisms of Cdc6p degradation are unknown, although the action of *CDC4* is required (177), suggesting the possible involvement of a PULC-type complex. Likewise, the ubiquitinylation and degradation of Cdc18 in the fission yeast requires the action of *pop1+*, a homolog of *CDC4* (159).

Transcription Factors, Tumor Suppressors, and Oncoproteins

The activity of many short-lived regulatory proteins of the transcription factor, tumor suppressor, and oncoprotein classes is controlled by proteolysis via the ubiquitin pathway. This section discusses some selected cases.

NF-κB AND IκBα NF-κB (nuclear factor κB) is a ubiquitous inducible transcription factor involved in central immune, inflammatory, stress, and developmental processes (reviewed in 179, 180). The best-studied transcription factor is a heterodimer, NF-κB1, that is composed of p50 and p65 (RelA). Similar complexes containing other subunits such as p52 (p52-p65 complex is NF-κB2) and RelB also exist. p50 and p52 are the processing products of larger precursor molecules, p105 and p100, respectively, and are derived from the N-terminal domain of the precursor molecules: The C-terminal domain is degraded. The processed transcription factor is retained in a latent form in the

cytoplasm of nonstimulated cells via association with inhibitory molecules collectively termed IκBs (inhibitors of κB). Following exposure of the cell to a variety of extracellular stimuli such as cytokines, viral and bacterial products, and stress, IκBs are degraded rapidly and the active heterodimer is translocated into the nucleus where it exerts its transcriptional activity. The inhibitors fulfill their task via a dual action: They sterically hinder the nuclear localization signal of the NF-κB proteins, thus retaining them in the cytosol, and they also inhibit their DNA binding and transactivation capacity. Recent evidence implicates the ubiquitin system both in the processing of p105 (and probably p100) and in the signal-induced degradation of IκBα (and probably IκBβ).

Fan & Maniatis (181) showed that p50 is generated in vivo by processing of the precursor protein p105. Reconstitution of a cell-free system using a truncated form of the precursor protein, p60, has revealed that the process requires ATP and is sensitive to N-ethylmaleimide, which inactivates E1, E2, and certain E3 enzymes. These findings suggest that the process may be mediated by the ubiquitin system. Palombella & colleagues (182) showed that COS cells transfected with p105 process the precursor protein to p50 and that processing can be inhibited by proteasome inhibitors. Experiments with yeast mutant cells defective in Pre1, one of the proteasomal subunits, provided direct evidence that processing is mediated by the proteasome. Reconstitution of a cell-free system has demonstrated that the processing requires ubiquitin (182), E2-F1 (or UbcH5 or UbcH7), and a novel species of E3 (82; see also above).

These findings establish a role for the ubiquitin system in the processing of p105. Since p105 is the only molecule known to be processed by the ubiquitin system rather than being completely destroyed, an obvious problem concerns the underlying mechanism(s) involved. Lin & Ghosh (183) have reported 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 stop-transfer signal that prevents processing of p105. Removal of GRR inhibits processing. A chimera protein constituted of p50, GRR, and IκBα was processed to yield p50 and IκBα, suggesting that generation of p50 from p105 proceeds in a two-step mechanism, a single endoproteolytic cleavage of p105 to generate p50 and the C-terminal domain, followed by degradation of the C-terminal part by an as-yet-unknown mechanism. Insertion of the motif between two unrelated proteins, gp10 and GST, yielded two authentic processing products, suggesting that the GRR can serve as a transferable stop processing signal.

Other experiments (A Orian & A Ciechanover, unpublished results), however, could not substantiate all of these conclusions. For example, insertion of GRR into Dorsal, the noncleavable *Drosophila melanogaster* p105 homolog, did not lead to processing of the protein. Insertion of GRR between p50 and ornithine decarboxylase (ODC) results in processing, but the ODC moiety is completely

degraded even in the absence of antizyme. Thus it appears that certain steric constraints determine recognition for processing and its mechanism, and that GRR clearly is not a universal transferable processing element. As for the mechanism of action of GRR, it may generate a structure that cannot enter the 26S proteasome. For example, insertion of a similar repeat at the C-terminal domain of Epstein-Barr nuclear antigen 4 (EBNA4) prevented its degradation but not its conjugation (184; see also below).

The IκB family of proteins contains, among other proteins, four inhibitors: IκBα, IκBβ, IκBε, and Cactus (179, 185). Most stimuli that induce NF-κB activation target the inhibitors for degradation. Signal-induced phosphorylation at specific sites directs IκB proteins for degradation via the ubiquitin system. When either Ser^{32} or Ser^{36} of IκBα were mutated, the inhibitor did not undergo stimulation-induced phosphorylation, was not degraded, and NF-κB could not be activated in response to a broad array of stimuli (186, 187). The S32E/S36E double mutant was constitutively unstable and NF-κB was constitutively hyperactive, suggesting a role for the negatively charged phosphate groups in the recognition process.

Additional studies have further indicated that phosphorylation targets the protein for degradation by the ubiquitin system. Incubation of TNF-α or phorbol ester/ionomycin-stimulated Jurkat T-cells in the presence of proteasome inhibitors results in stabilization of the otherwise rapidly degraded phosphorylated IκBα and in accumulation of high-molecular-mass ubiquitin conjugates of the protein (188–190). Reconstitution of a cell-free system revealed an excellent correlation with the in vivo findings. Only the doubly phosphorylated form, but not the S32A/S36A double mutant or singly mutated S32A or S36A species, could be ubiquitinylated in vitro. Similarly, the S32E/S36E double mutant could be ubiquitinylated in vitro (188). Reconstitution of degradation revealed that the process requires ATP, ubiquitin, and the E2 enzymes E2-F1, which is the rabbit homolog of human UbcH7 (see above) or Ubc5.

In ts20 cells that harbor a thermolabile E1, phosphorylated IκBα was stable at the nonpermissive temperature (189). Addition of purified 26S proteasome to immunopurified IκBα ubiquitin conjugates led to their degradation (188). The ubiquitinylation sites have been localized to Lys residues 21 and 22 (191). These findings strongly link stimulation-induced phosphorylation at Ser residues 32 and 36 to recognition by the ubiquitin-conjugating machinery and to subsequent degradation by the proteasome. It is important to note that phosphorylation does not release IκBα from the NF-κB complex: it is degraded while still bound to the complex (188, 192).

An interesting problem involves the mechanism of phosphorylation-dependent targeting of IκBα. Is the phosphorylated domain [32-S(P)GLDS(P)-36] recognized directly by the IκBα-ubiquitin ligase, or does phosphorylation affect

the 3-D structure of the inhibitor indirectly in a manner that exposes a remote E3-binding site? Yaron et al (193) have shown that phosphorylated peptides that span the phosphorylation domain, but not their unmodified or mutated counterparts, specifically inhibit conjugation and degradation of IκBα in a cell-free reconstituted system. A seven-residue peptide [31-DS(P)GLDS(P)M-37] is sufficient to exert the inhibitory effect. Notably, Lys residues 21 and 22 are dispensable and do not constitute a part of the recognition signal. Incubation of a crude extract with immobilized peptide leads to specific binding of a conjugating activity that can be complemented only by the addition of an E3-rich fraction, but not by E1 and E2. Microinjection of these phosphopeptides into cells leads to inhibition of translocation of NF-κB to the nucleus and, consequently, to inhibition of expression of the NF-κB-dependent gene, *E-selectin*. These results suggest that the phosphorylated domain serves as recognition motif for an E3 that acts on IκBα and that inhibition of this E3 by the mimetic peptides can inhibit the biological functions of NF-κB.

Two kinases that phosphorylate IκBα have been cloned recently and were identified as the previously known Ser-Thr kinase of unknown function, CHUK, and a close homolog of this protein (194, 195, 195a). CHUK associates directly with IκBα and phosphorylates it on Ser residues 32 and 36. The direct or indirect role(s) of other kinases that have been reported to be involved in NF-κB activation via phosphorylation of IκBα, such as the mitogen-activated protein kinase/ERK kinase kinase 1 (MEKK1; 196), the protein kinase A catalytic (PKAc) subunit (197), and an unidentified novel kinase that appears to be activated by ubiquitinylation (198) are not clear. At least for CHUK, there is no evidence that it is activated by ubiquitinylation.

P53 The tumor suppressor protein p53 is degraded by the ubiquitin system both in vitro (199) and in vivo (200). Its degradation in vitro requires E1 and is blocked in intact cells by inhibitors of the proteasome with the concomitant accumulation of high-molecular-mass p53-ubiquitin adducts. Degradation is accelerated dramatically by the high-risk human papilloma virus (HPV) oncoprotein E6 (53). The E6-dependent degradation is mediated by the E3 enzyme E6-AP and by one of several E2 enzymes (see previous section).

Since wild-type p53 is short lived in all cells examined, and since it is targeted for degradation by the ubiquitin system, an important problem involves the identity of the ubiquitin pathway enzymes (E2 and E3), which conjugate p53 in cells that do not express E6. Antisense targeting of E6-AP results in elevated p53 levels in HPV-infected cells but not in normal cells (201), suggesting that E6-AP plays a role in targeting of p53 only in the presence of E6. In most cells that are not transformed by HPV, ubiquitinylation of p53 is mediated by an unidentified species of E3.

Haupt et al (202) and Kubbutat et al (203) have reported that Mdm2 promotes rapid, ubiquitin-dependent degradation of p53. The oncoprotein Mdm2 is a potent inhibitor of p53. It binds to the transcriptional activation domain of p53 and inhibits its ability to activate target genes, to exert its antiproliferative effects, to mediate cell-cycle arrest following exposure to DNA damage, and to fulfill its apoptotic functions. p53 regulates the transcription of Mdm2 in an autoregulatory feedback loop (202 and references therein). The transient stabilization of p53 that occurs following UV irradiation and DNA damage, for example, leads to an increase in its level, which enables the tumor suppressor protein to curb the damage. Concomitantly, increased, p53-dependent transcription of Mdm2 ensures, by targeting p53 for degradation, release from the cell-cycle arrest and other untoward effects caused by elevated p53 level. Thus the targeting of p53 for degradation by Mdm2 provides another way to remove p53 after its repair functions have been fulfilled.

The interval between p53 stabilization and activation and its inactivation and targeting by induced Mdm2 defines a time window for its activities. Induction of degradation is accompanied by accumulation of high-molecular-mass p53-ubiquitin adducts and can be inhibited by lactacystin. The destabilizing effect of Mdm2 requires physical interaction between the two proteins. This interaction is mediated by a small region in the N-terminal domain of p53. Fusion of amino acid residues 1–42 of p53 to Gal4, an otherwise stable protein, renders the chimeric protein susceptible to Mdm2-mediated degradation (202). Thus the N-terminal domain of p53 involved in Mdm2 binding is necessary and sufficient to confer upon the tumor suppressor Mdm2-dependent proteolytic sensitivity. It remains to be seen whether, like E6, Mdm2 is part of an E3 complex that targets p53 for degradation.

JUN Treier et al (105) reported that c-Jun, but not its transforming counterpart v-Jun, is multiply ubiquitinylated and rapidly degraded in cells. Detailed analysis of the differential sensitivity to the ubiquitin system revealed that the δ domain of c-Jun, an amino acid sequence that spans residues 31–57 and that is missing in the retrovirus-derived molecule, confers instability on the normal, cellular protein. Deletion of the domain stabilizes c-Jun. This requirement for the δ domain raised the question of whether it contains either the ubiquitinylation site or a recognition signal for the conjugation machinery. Alteration of all the Lys residues in the domain did not alter the protein's sensitivity to degradation, and no single Lys residue in the remaining parts of the molecule was essential for ubiquitinylation. Thus, the δ domain is a *cis*-acting signal required for ubiquitinylation and subsequent degradation of c-Jun. Transfer of this element to β-Gal, an otherwise stable protein, rendered the protein susceptible to multiple ubiquitinylation and degradation.

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 the possible resulting transforming activity of v-Jun. The loss of the δ domain during the retroviral transduction is another example of the sophisticated diverse mechanisms developed by viruses to ensure replication and continuity of infection (see above for the effect of E6 on targeting p53 and below for the effect of the cytomegalovirus proteins US2 and US11 on targeting MHC class I heavy chains). Musti et al (94) showed that phosphorylation by mitogen-activated protein kinases (MAPKs), such a Jun kinase 1 (JNK1), reduces c-Jun ubiquitinylation and increases its stability. c-Jun^{Asp}, in which one of the Ser targets of JNK1 was substituted by the phosphate-mimetic residue, Asp, acts as a gain of function form of c-Jun. It is not ubiquitinylated and, consequently, remains stable. In contrast, c-Jun^{Ala} is as efficiently ubiquitinylated as the wild-type protein. Thus, it appears that phosphorylation-dependent stabilization contributes to the efficient activation of target genes following exposure to growth factors, stress, or other stimulators of c-Jun activity. In another study, Fuchs et al (204) showed that JNK2 can, in addition, stimulate ubiquitinylation of c-Jun, underscoring the complexity of the signals and extracellular events that govern c-Jun stability.

β-CATENIN β-Catenin and its *D. melanogaster* homolog, Armadillo, are multifunctional proteins involved in cell-cell adhesion complexes, signal transduction along the wingless (*D. Melanogaster*)/Wnt-1 (mammals) pathway, and regulation of transcription (reviewed in 205). Accordingly, the protein is found in the plasma membrane, cytoplasm, and nucleus. The wingless/Wnt-1 pathway is involved in several key developmental processes, such as in determining anterior-posterior patterning in *Drosophila* segments and axis formation in *Xenopus*. Its specific expression in early mouse embryos implicates the protein in the development of mammals as well. Genetic and biochemical analyses showed that β-catenin plays a major role in signal transduction and differentiation in the mammalian colorectal epithelium, and that aberrations in the process are important in the multistep development of colorectal tumors.

In the absence of signaling by wingless/Wnt-1 ligands, the downstream *Drosophila* Ser/Thr kinase zeste white 3 (zw3), or its mammalian homolog glycogen synthase kinase-3 (GSK-3), is active and promotes degradation of β-catenin, most probably by ubiquitinylation (90). The cadherin-associated β-catenin in the cell-cell junctions remains stable. Stimulation by wingless/Wnt-1 promotes activation of the cytosolic protein disheveled (dsh), which antagonizes the function of zw3/GSK-3, leading to stabilization and accumulation of β-catenin. Free β-catenin binds and activates transcription factors Tcf (T-cell transcription factor) and Lef (lymphoid enhancer factor). The translocation of

the active complexes to the nucleus initiates transcription of specific, yet-to-be-identified target genes.

β-Catenin interacts with both GSK-3 and an additional protein, the ~300-kDa tumor suppressor APC (adenomatous polyposis coli), which appears to regulate β-catenin intracellular level (206). GSK-3 phosphorylates β-catenin in three conserved Ser residues and one Thr residue in the N-terminal domain (33-SGIHSGATTTAPS-45). Mutations of these residues (S33Y, S37F, S45Y), or deletions of the N-terminal domain containing these residues in different malignant melanoma cell lines, result in increased stability and consequent increased activity of the protein (207, 208). The constitutive high levels of β-catenin-Tcf and β-catenin-Lef complexes in these conditions may result in persistent *trans*-activation of the target gene(s) and may play a crucial role in malignant transformation of these cells.

Phosphorylation-dependent degradation of β-catenin is another example in which this posttranslational modification destabilizes a protein (see above for other examples). It is unknown how APC regulates the stability of β-catenin; however, APC interacts with β-catenin via two distinct sets of binding sites. β-catenin accumulates in colon cancer cells that do not express the protein ($APC^{-/-}$) or that harbor APC proteins that lack one of the binding clusters. The accumulated β-catenin associates with Tcf or Lef and leads, most probably, to overexpression of their dependent genes (209). Expression of full-length APC in these cells leads to degradation of excess β-catenin and to abrogation of the *trans*-activation effect. Although the mechanism(s) that underlie the function of APC in regulating β-catenin stability have not been elucidated, it may serve as a ubiquitin ligase or as part of a larger complex that serves as a ligase. Indeed, wild-type but not ΔN1-89 β-catenin is part of a large complex that contains, among other proteins, APC (210).

E2F-1 The E2F family of transcription factors plays an important role in regulating cell-cycle progression at the G_1-S transition. One of the best studied members of this family is E2F-1, a 437-amino-acid residue protein that has a half life of approximately 2–3 h. E2F-1 can act under different circumstances as an oncogene or as an inducer of apoptosis and is controlled by multiple mechanisms, among which are binding to the retinoblastoma tumor suppressor (Rb) protein, activation by Cdk3, and S-phase-dependent down-regulation of its DNA-binding capacity by cyclin A–dependent kinase. E2F-1 is degraded in a regulated manner by the ubiquitin system (211, 212). In the presence of proteasome inhibitors, the protein was stabilized with the concomitant accumulation of high-molecular-mass E2F-1-ubiquitin adducts. Co-expression of Rb with E2F-1 results in a marked stabilization of E2F-1. The stabilization is the result of direct binding of Rb to the transcription factor, as deletion of either

the E2F-1-Rb- or the Rb-E2F-1-binding sites abrogated the stabilizing effect of Rb.

Mechanistically, binding of Rb prevents ubiquitinylation of E2F-1. The dephosphorylated form of Rb binds E2F-1, as a mutant form of Rb that could not undergo phosphorylation was much more efficient in stabilizing E2F-1 than its wild-type counterpart. An interesting problem involves the cell-cycle regulation of E2F-1 and its linkage to Rb binding. Hofmann et al (211) noted a cell-cycle oscillation in the level of E2F-1. In particular, there appeared to be a sharp decrease during S-phase. Whether the changes reflect cell-cycle-dependent dissociation from Rb and degradation of free E2F-1 remains to be examined.

Membrane Proteins

It has been generally accepted that the ubiquitin system is involved in selective degradation of cytosolic and nuclear proteins. However, it is clear now that the system is also involved in two distinct pathways of degradation of membrane proteins. A new and rather unexpected function of ubiquitin tagging was found in targeting some membrane receptors and transporters for endocytosis and degradation in the lysosome (or the vacuole in yeast). In a distinct pathway, native or misfolded proteins in the endoplasmic reticulum (ER) are targeted to the proteasome and degraded in the cytosol (or on the cytosolic surface of the membrane), either with or without prior ubiquitinylation. These novel findings raise important, yet unresolved, mechanistic issues. For membrane proteins an obvious problem relates to the role of ubiquitin modification: Is it required for endocytosis of the tagged protein or in a later stage, for its specific targeting and uptake by the lysosome? For membrane ER proteins degraded by the cytosolic proteasome, important questions involve the mechanisms that underlie retrieval of these proteins across the membrane back into the cytosol.

Analysis of the fate of the *S. cerevisiae* Ste2p, the G-protein-coupled plasma membrane receptor of the α factor involved in the mating response pathway, has shown that binding of the ligand leads to ubiquitinylation of the receptor that is essential for endocytosis of the receptor-ligand complex (213, 214). The complex is targeted for degradation in the vacuole: In mutant yeast species lacking vacuolar proteolytic enzymes, the receptor is stable. These results suggest that both the ectoplasmic and cytoplasmic portions of the molecule are degraded within the vacuole. Binding of the α factor leads to phosphorylation of the receptor on Ser residues that reside on a well-defined internalization signal, SINNDAKSS, that is both necessary and sufficient for receptor endocytosis (215, 216). The Lys residue in this sequence is ubiquitinylated, though it is dispensable: Other adjacent Lys residues can also bind ubiquitin. Detailed structural analysis of the SINNDAKSS sequence showed that receptor

ubiquitinylation is necessary for internalization of the ligand-receptor complex: Inhibition of ubiquitinylation results in stabilization of the receptor on the cell surface as shown by radiolabeled ligand binding assays. Interestingly, endocytosis of the Ste6 ABC transporter also requires ubiquitinylation and a DAKTI motif (217) that is similar to the essential SINNDAKSS sequence in the Ste2p receptor. In addition, Pdr5, the yeast multidrug transporter, is also targeted to the vacuole by ubiquitinylation (218).

Using a Chinese hamster cell-cycle mutant cell that harbors a thermolabile E1, Strous et al (219) showed that following binding of growth hormone (GH) at the permissive temperature, the receptor of GH undergoes rapid ubiquitinylation and subsequent degradation in the lysosome: Inhibitors of endosomal/lysosomal function, such as NH_4Cl and bafilomycin A1, significantly inhibited ligand-induced degradation of the receptor. In contrast, at the nonpermissive temperature, the receptor was not ubiquitinylated and remained stable. Similar to the case of the Ste2p receptor, these findings establish a direct linkage between ubiquitinylation and early events in endocytosis of ligand-receptor complexes in mammalian cells. The exact role of ubiquitinylation in the endocytic process is unclear. It may serve, for example, as an anchoring site for adaptor molecules or cytoskeletal elements involved in vesicle budding and movement. It can also signal limited proteolysis of the cytosolic tail by the proteosome that is necessary for initiation of the endocytic process to occur.

The yeast Gap1p amino acid permease and the Fur4 uracil permease are also targeted for degradation in the vacuole following ubiquitinylation that is mediated by the E3 enzyme Npi1p/Rsp5 (61, 62; see also previous section). Ubiquitinylation of the two proteins is triggered by NH_4^+ or stress conditions (such as approach to the stationary growth phase or inhibition of protein synthesis), respectively. Recent findings demonstrate that ubiquitinylation of the Fur4 protein, and probably of other membrane proteins, is mediated by a mechanism that is distinct from that of tagging soluble proteins. Polyubiquitinylation and endocytosis of the protein were inhibited in a yeast mutant lacking the deubiquitinylating enzyme Doa4 that prevents the regeneration of free ubiquitin. Interestingly, both processes could be rescued by overexpression of ubiquitin mutants carrying Lys $\rightarrow$ Arg mutations at Lys^{29} and Lys^{48}. By contrast, a ubiquitin mutated at Lys^{63} did not restore Fur4 polyubiquitinylation but interfered only slightly with endocytosis (220).

Similar findings were reported for the endocytosis of the α factor receptor (220a). It appears, therefore, that ubiquitin-Fur4 and ubiquitin-Ste2p conjugates are extended via Lys^{63}, but this process is not essential for endocytosis: Monoubiquitinylation is sufficient to drive the receptors into the vacuolar system of the cell. Another yeast membrane protein, the galactose transporter

Gal2p, is ubiquitinylated, endocytosed, and targeted to the vacuole following transfer of cells from a galactose- to a glucose-containing medium (221). Down-regulation of the Kit receptor by its ligand, the soluble steel factor (SSF), is also mediated by ubiquitinylation that targets the endocytosed receptor to the lysosome (222). Down-regulation of this receptor, involved in hematopoiesis, melanogenesis, and gametogenesis, requires the kinase activity of the molecule, although surprisingly, substitution of Tyr^{821}, which undergoes autophosphorylation, does not affect the process (223). Thus the role of the receptor kinase in the endocytic process is still obscure. It was reported that angiotensin II down-regulates the inositol triphosphate receptors via the proteasome, but it is unclear whether ubiquitinylation is required in this process (224).

The involvement of the ubiquitin-proteasome pathway in different aspects of membrane protein degradation has highlighted the role the system plays in a variety of pathophysiological processes in the immune system. The ζ subunit of the T-cell receptor (TCR), a disulfide-linked homodimer that plays an important role in TCR-mediated signal transduction, also undergoes polyubiquitinylation in response to receptor engagement (225). This modification can occur on multiple Lys residues (226) and requires phosphorylation of the cytosolic tail of the chain by the tyrosine kinase $p56^{lck}$ (226). A tyrosine phosphatase that regulates the activity of the kinase is also required in the process (227). The TCR is a long-lived protein, and it is unclear whether ubiquitinylation plays a role in modulating TCR activity via degradation of a single subunit, or whether it is required for the function of the receptor in signal transduction.

While most cell surface receptors appear to be targeted to the lysosome/vacuole, several exceptional cases were reported in which ubiquitinylation targets membrane proteins to the proteasome. For example, the platelet-derived growth factor receptor is ubiquitinylated following ligand binding and is degraded by a process partially inhibited by proteasome inhibitors (228). In addition, the Met tyrosine kinase receptor involved in pleiotropic cellular response following activation by its ligand, the hepatocyte growth factor/scatter factor (HGF/SF; see 229), and the gap junction protein connexin43 (230) are ubiquitinylated and degraded in a similar fashion. Ubiquitinylation of other cell surface receptors such as the high-affinity IgE receptor (231), the prolactin receptor (232), and the EGF receptor (233), has been reported; however, the function of the modification in regulating the level and activity of these molecules and the underlying mechanisms involved are still obscure.

Thus, an emerging view is that rapid ligand-induced endocytosis of certain cell surface receptors requires ubiquitinylation of their cytosolic tails. The role of ubiquitinylation in the endocytic process is not known and may involve proteolysis of the tail: Formation of multivesicular, multilayered bodies as transport intermediates en route to the lysosome appears to be essential for

degradation of both the ectoplasmic and the cytosolic domains of these proteins in the vacuole/lysosome system. Unlike the degradation of lumenal ER proteins, proteolysis of membrane-anchored proteins in the lysosome does not require their transport across the membrane into the cytosol.

The ER is the port of entry of most compartmentalized, membrane-anchored, and secretory proteins. It is also the site of folding and modification of nascent chains and of assembly of multisubunit complexes. Therefore, it must be endowed with a "quality control," editing machinery to remove proteins that fail to fold properly or to oligomerize. Such proteins can include aberrant, mutated proteins or excess subunits of large complexes. Some resident ER proteins are under tight physiological control, and their degradation must also be regulated by a highly specific machinery, distinct from the lysosome. Until recently, degradation of ER proteins was thought to involve an unidentified ER-localized protease(s)/proteolytic system (234). It has become clear, however, that this is not the case, and that ER degradation represents a novel type of ubiquitin-mediated degradation of membrane proteins, distinct from that of mature cell surface proteins. Degradation of these proteins occurs in the cytosol and is mediated by the 26S proteasome, with or without prior ubiquitinylation (for recent reviews, see 235, 236). Unlike ubiquitin-induced targeting of cell surface proteins to lysosomes, degradation of ER proteins requires retrograde transport of these proteins back into the cytosol. Here the function of ubiquitinylation, when modification occurs, is known, and it most likely serves in targeting to the proteasome.

An important ER membrane protein is 3-hydroxy-3-methylglutaryl-coA reductase (HMG-R), a key regulatory enzyme in sterol synthesis. The mammalian enzyme has several transmembrane domains and is tightly regulated by the final product of the biosynthetic pathway, cholesterol. Regulation is mediated by both feedback inhibition and rapid targeting for degradation. Yeast has two isozymes, Hmg1p and Hmg2p. The regulation of the first enzyme occurs mostly at the translational level, whereas that of Hmg2p is mediated in an manner identical to the regulation of the mammalian enzyme, via end-product inhibition and induced degradation. Recent evidence implicates the ubiquitin-proteasome pathway in the degradation of Hmg2p in yeast. Degradation proceeds independently of vacuolar proteases (237). Genetic analysis has revealed that the proteolytic process requires the product of *HRD2* (HMG-R degradation), which is a subunit of the 19S regulatory complex of the proteasome and is homologous to the p97/TRAP2 19S subunit in the mammalian proteasome (238).

A recent study showed that the enzyme undergoes Ubc7-mediated polyubiquitinylation prior to its degradation (239). In contrast, while the mammalian enzyme is also degraded by the proteasome as shown by inhibition of sterol-induced degradation by lactacystin, it does not appear to require prior

ubiquitinylation (240). However, genetic analysis in mammalian cells is more difficult than in yeast, and the conclusion that ubiquitinylation is not involved in the degradation of a certain protein is based solely on experiments with a temperature-sensitive mutant of E1, which is difficult to inactivate completely (241).

The cystic fibrosis transmembrane conductance regulator (CFTR) is synthesized as an approximately 140-kDa core glycosylated protein that is associated initially with the cytosolic chaperone Hsc70 and then with the ER chaperone calnexin. Only 25–50% of the wild-type protein matures to the cell surface, whereas most of the protein molecules do not fold properly and are degraded in the ER (242, 243). A single mutation in the protein, deletion of Phe^{508}, is the underlying cause of most cases of cystic fibrosis. The mutated protein is never released from its complex with the chaperone, fails to acquire carbohydrate moieties (a hallmark of transit through the Golgi apparatus), and is degraded. Recent evidence suggests that the process is mediated by the ubiquitin-proteasome pathway. Lactacystin inhibits degradation of both the wild-type and the mutant forms of CFTR. Degradation is also inhibited in a mutant cell line harboring a thermolabile mutation in E1 and in cells that express a dominant negative form of ubiquitin (244, 245). These data suggest that ubiquitinylation precedes recognition and degradation of the wild-type and mutant CFTR proteins by the proteasome.

Degradation of TCR subunits in the ER constitutes an important mechanism that ensures that only correctly assembled receptor complexes will reach the cell surface. The CD3-δ subunit of the TCR is degraded in the ER following ubiquitinylation (246). Inhibition of the proteasome by lactacystin leads to accumulation of an insoluble, membrane-bound form of CD3-δ, suggesting that the ubiquitin system selectively degrades a misfolded denatured form of the protein. The misfolded form is generated, most probably, because of the inability of the excess unassembled chains to incorporate into the mature TCR complex. A more detailed study of the mechanism revealed that the process initiates with the trimming of mannose residues from an N-linked oligosaccharide, generation of membrane-bound ubiquitin conjugates, and removal of these conjugates by the proteasome. The α chain of the TCR is degraded via a similar mechanism, except that ubiquitinylation does not appear to mediate recognition by the proteasome (247, 248). Again, similar to the case of mammalian HMG-R, the lack of requirement for ubiquitinylation is not firmly substantiated.

Antigenic peptides that are processed in the cytosol are presented to cytotoxic T-cells on MHC class I complexes. These complexes are heterodimers constituted of transmembrane heavy chains (HCs) associated noncovalently with β_2-microglobulin (β_2-m). The proper folding of the complex is aided by the ER chaperones calnexin and calreticulin. If β_2-m is absent, the complex will

not be assembled and antigenic peptides will not be transported and presented on the cell surface. Inhibition of the proteasome in β_2-m-deficient cells leads to accumulation of de-glycosylated HCs in the cytosol (249). These molecules that were transported into the ER membrane and glycosylated cannot fold properly and are transported back to the cytosol, de-glycosylated, and degraded by the proteasome.

An interesting observation involving the stability of MHC class I complexes relates to the multiple mechanisms by which viruses evade the immune surveillance machinery. One such mechanism involves a viral matrix protein–directed phosphorylation of the immediate early (IE) protein of the cytomegalovirus (CMV) that prevents its processing to peptide antigens and, consequently, their presentation to cytotoxic T-cells (CTLs; see 250). Since the proteasome is involved in processing of antigens presented via class I MHC molecules (see below), it is assumed that inhibition of processing involves interference with recognition by the proteasome.

In a different mechanism, researchers noted that the human CMV encodes two ER resident proteins, US2 and US11, that down-regulate the expression of MHC class I HC molecules. The MHC molecules are synthesized on membrane-bound ribosomes, transported to the ER where they are glycosylated, but shortly thereafter, in cells expressing US2 or US11, are transported back to the cytosol, de-glycosylated by N-glycanase, and degraded by the proteasome (251, 252). It appears that the viral products bind to the MHC molecules and escort/dislocate them to the translocation machinery where they are translocated back into the cytosol in an ATP-dependent process. The mechanism of action of the viral proteins is not known. They may diffuse laterally in the membrane, interact with the emerging nascent MHC chain, and not allow insertion of the stop-transfer signal and proper anchoring of the molecule in the membrane. Alternatively, they may compete with the binding of the ER chaperone Bip/kar2, which may be necessary for proper folding of the HC molecule. In a different case, ICP467, an IE cytosolic protein encoded by the herpes simplex virus, prevents transport of cytosolic peptides into the ER lumen. Expression of this viral protein also leads to rapid degradation of MHC HCs (249). The lack of peptides can lead to changes in the conformation of otherwise intact and properly folded MHC complexes. Consequently, they are retrieved from the ER and degraded in the cytosol by the proteasome.

Carboxypeptidase Y (CPY) is a yeast vacuolar enzyme that, like all other vacuolar enzymes, traverses the ER lumen en route to the vacuole. Mutated CPY is degraded rapidly and never matures to the vacuole (253). Recent evidence suggests that it is ubiquitinylated and degraded by the proteasome in the cytosol (254). In clear distinction from the MHC molecules that are membrane-anchored proteins, CPY is a soluble lumenal ER protein. Thus, following

synthesis, glycosylation, and *complete* transport to the ER lumen, it has to bind to the lumenal face of the ER membrane and be translocated into the cytosol for ubiquitinylation and degradation.

The notion that ER proteins are translocated in a retrograde manner raises several important mechanistic problems. One problem involves the specificity of recognition and the ability of the dislocation machinery to distinguish between properly folded and misfolded substrates. It is assumed that the proper folding of a protein requires a chaperone, and if the chaperone "fails" to fold a protein in repeated ATP-dependent association-dissociation cycles, it presents it to the ubiquitin proteolytic machinery (the "refold or degrade" function of chaperones; see 255). Such a mechanism has been described for soluble proteins (256, 257). The process in the ER probably involves chaperones as well. Indeed, the dislocation of CPY requires the Bip/Kar2 chaperone (258). The chaperones involved in folding of normal proteins must distinguish between normal proteins in the process of folding and misfolded proteins destined for degradation. The underlying mechanisms involved in signaling one population from the other are not known. The viral proteins US2 and US11 probably act as chaperones as well; however, they are different from the cellular native chaperones in the sense that they target a normal, properly folded (or in the process of folding) protein for degradation.

Another problem involves the machinery through which transport occurs. It appears that the retrograde transport is mediated by the Sec61 complex, which is also involved in anterograde insertion and transport of proteins into the ER membrane and lumen. In US2-expressing cells, de-glycosylated MHC molecules were immunoprecipitated along with components of Sec61 (252). Similarly, retrograde transport of mutated CPY and other misfolded secretory soluble proteins also require the yeast Sec61 translocation complex (258, 259). Interestingly, degradation of misfolded mutant components of the Sec61 complex requires the E2 enzymes Ubc6 and Ubc7 and is mediated by the proteasome (260). Since Ubc7 is a soluble cytosolic protein, it has to be recruited to the membrane, a process mediated by a novel protein, Cue1p (260a). Finally, dislocation requires energy. Indeed, US2-mediated targeting of the HCs of the MHC complex is ATP dependent. The cytosolic face of the ER membrane contains several candidate ATPases that could provide the energy necessary for dislocation. These include the cytosolic chaperone Hsc70 and the 19S regulatory complex of the 26S proteasome.

DIVERSE FUNCTIONS OF THE UBIQUITIN SYSTEM

Recent evidence implicates the involvement of the ubiquitin pathway in a variety of basic cellular processes. In many cases evidence is still circumstantial, and

neither the cellular targeted substrates nor the underlying mechanisms involved have been elucidated. Yet, the processes appear to be important and therefore deserve special attention.

DEVELOPMENT The involvement of the ubiquitin system in human brain development is indicated by a defect in a gene coding for the E3 enzyme E6-AP, which has been implicated as the cause of Angelman syndrome, a disorder characterized by mental retardation, seizures, and abnormal gait (56, 57; see also above). Other evidence links the ubiquitin system to developmental processes in the central nervous system (CNS). The *Drosophila bendless* (*ben*) gene encodes an E2 that appears to be restricted to the CNS during development. Mutations in this gene lead to morphological abnormalities within the visual system that involve, for example, impairment of synaptogenesis between photoreceptor cells and other elements of the system (10, 261). The mutation was described initially as a behavioral defect affecting the escape jump response and was ascribed to a lesion affecting the connectivity of the giant fiber to the motor neuron innervating the tergotrochanter muscle. It appears that the function of the gene product is broader and is involved in other developmental processes of the neuromuscular system as well.

Another gene that encodes a developmentally regulated de-ubiquitinating enzyme from the UBP (see above) family is the *Drosophila faf facets* (*faf*; see 130, 262). The *faf* gene is specifically required for eye development, and mutant *faf* flies have more than eight photoreceptors in each of the compound eye units, the facets. The *faf facets* 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. The only other defect found in *faf* mutants affects the eggs that do not reach cellularization during early embryogenesis. The target protein(s) of *faf facets* have not been identified so far (however, see below).

Another recently described link between the ubiquitin system and *Drosophila* eye development is related to the function of *Tramtrak* (TTK88), *Phyllopod* (PHYL), and *Seven in Absentia* (SINA). TTK88 is a zinc-finger embryonic transcription factor whose expression represses neuronal cell fate determination in the developing eye. PHYL, in a mechanism that also requires SINA, antagonizes the activity of TTK88. Activation of the Sevenless Ras/MAPK signaling cascade by the Sevenless receptor tyrosine kinase, known to be involved in cell differentiation, leads to transcriptional induction of PHYL. The induced PHYL, TTK88, and SINA generate a heterotrimeric complex that targets TTK88 for degradation (263). SINA was found to interact with UbcD1, a *D. melanogaster* E2 enzyme, and a mutation in *UbcD1* serves as a dominant suppressor of the effects caused by overexpression of the SINA protein. A

mutation in *faf facets* serves as a dominant enhancer for reduced *sina* activity (263). The role of SINA may be as an adaptor protein that provides the link between the TTK88/PHYL/SINA heterotrimer and the ubiquitin system (see above), whereas TTK88 may well be one substrate that is targeted by *faf facets*: *faf* may be an editing isopeptidase, protecting TTK88 from untimed degradation (see above). In another study, Li et al (264) showed that SINA and PHYL promote ubiquitinylation of TTK88, which is then degraded rapidly by the proteasome.

Roest et al (11) demonstrated that inactivation of HR6B, a human homolog of the yeast E2 RAD6/Ubc2 involved in DNA repair and targeting of N-end rule substrates (see above), leads to a single defect, male sterility: Knockout females are completely normal. The defect is specific to the development of sperm and does not involve the general process of meiosis. The lack of more severe effects may be due to the presence of a highly homologous enzyme, HR6A. The protein target of HR6B in the spermatids has not been identified. The authors hypothesized that HR6B is involved in polyubiquitinylation and degradation of histones. This process is critical for chromatin remodeling, which involves replacement of histones with transition protamines and, subsequently, with protamines. Histones are N-α-acetylated proteins, and HR6B may act on these proteins either via the non-N-end rule recognition site of E3 (see above) or via a novel, yet-to-be-identified species of E3. Another ubiquitin system–related gene involved in sex differentiation, oogenesis, or spermatogenesis is the *hyperplastic disc* (*hyd*) gene, which appears to play a major role in *D. melanogaster* development. The null phenotype appears to be lethality at an early embryonic stage; however, adults obtained by crosses of temperature-sensitive alleles and maintained at the permissive temperature are sterile and display, in addition to imaginal disc overgrowth, morphological abnormalities in the germ tissue (265). The gene encodes an approximately 280-kDa protein that belongs to the HECT family of E3 enzymes (see above). The target proteins of this E3 enzyme have not been identified.

APOPTOSIS During development, a large number of cells die in a predicted spatial and temporal pattern known as programmed cell death (PCD), or apoptosis. This process is crucial for differentiation and involves programmed regulation of gene expression. One of the first genes shown to be involved in PCD is the polyubiquitin gene that is up-regulated during the metamorphosis of the hawkmoth *Manduca sexta* (266). During the 30-h period that precedes the adult moth's emergence from the pupal cuticle, there is a rapid degradation of the large mass of intersegmental muscles that served the pupa. The resulting amino acids are probably used in building the wing muscles and also as a source of energy for the butterfly's short life span. The strong induction of transcription

of the polyubiquitin gene during the metamorphotic process is accompanied by a parallel increase in the level of ubiquitin. Qualitative and quantitative changes occur concurrently in the 26S proteasome (267, 268). Pools of ubiquitin conjugates increase 10-fold during this period, accompanied by an increase in the activity of E1, several E2 enzymes, and E3 enzyme(s) of unknown specificity (269). This coordinated induction of ubiquitin conjugation and degradation pathways is stimulated by the programmed decline in the steroid-like molting hormone, 20-hydroxyecdisone. All the changes can be blocked by administration of the hormone.

Several other studies have linked the ubiquitin system to apoptosis in different systems; however, because of the complexity and variety of the apoptotic pathways, it is unclear whether the system plays a primary causative or only a secondary role in the process. Also, the system's protein targets in these processes remain unidentified. The complexity of the processes is also reflected in the apparently conflicting reports as to the system's role in apoptosis in different experimental systems. In some model systems, PCD requires the activity of the ubiquitin pathway, whereas in others PCD is invoked following inhibition of the ubiquitin system.

γ-irradiation-induced apoptosis in human lymphocytes is accompanied by increased ubiquitin mRNA and ubiquitinylated nuclear proteins. Expression of ubiquitin sequence-specific antisense oligonucleotides leads to a significant decrease in the proportion of cells displaying the apoptotic phenotype (270). Similarly, lactacystin prevents ionizing irradiation-induced cell death of thymocytes (271). Inhibition of the ubiquitin system leads to prevention of apoptosis induced by NGF deprivation in sympathetic neurons (272). These findings suggest an active role for the ubiquitin system in PCD. However, in leukemic cells (273), activated T-cells (274), and some neuronal cells (275), inhibition of the ubiquitin system stimulates apoptosis. Thus, the involvement of the system appears to be cell- and environment-specific. In some cases inhibition of the system may lead to the accumulation of abnormal proteins with the possible consequent induction of apoptosis, whereas in others the system may play a direct role in the destructive process and its inhibition leads to an inhibition or a delay in the onset of the apoptotic chain of events.

ANTIGEN PROCESSING Peptide epitopes presented to cytotoxic lymphocytes (CTLs) on class I MHC molecules are generated in the cytosol by limited processing of antigenic proteins. Although it was reported that the process can be mediated by ubiquitinylation- (276, 277) or proteasome-independent (278) mechanisms, it appears that many MHC class I antigens are processed by the ubiquitin-proteasome pathway (for review, see 4, 279).

Rock et al showed that both the less specific peptide aldehyde inhibitors (122) and the more specific proteasome inhibitor, lactacystin (280), inhibit processing and presentation of class I MHC antigens. The cytokine γ-interferon (γ-IFN) that stimulates antigen presentation leads also to induction and exchange of three proteasomal subunits in human cells: LMP2 for X (MB1, ε), LMP7 for Y (δ), and MECL1 for Z (281, 282). These exchanges lead to alteration in the cleavage site preferences of the proteasome: The tryptic- and chymotryptic-like activities are stimulated, whereas the PGPH activity is decreased. The changes in activities, which are mostly the result of the newly incorporated subunits LMP2 and LMP7, result in peptides that terminate mostly with basic and hydrophobic residues, similar to the vast majority of known peptides presented on MHC class I molecules. These C-termini may be required for selective uptake by the ER TAP transporter (283) and for better binding to the MHC molecule (284).

Initial reports showed that LMP2 and LMP7 are not essential for antigen presentation (285, 286). However, more detailed quantitative analyses showed that cells lacking these subunits have a decreased efficiency rather than an absolute inability to present antigens (287, 288). Macrophages and spleen cells derived from knockout mice lacking LMP2 exhibit a reduced capacity to stimulate a T-cell specific for a nucleoprotein epitope of the *Haemophilus influenza* A virus (288). The mutant mice themselves have significantly reduced levels of CD8+ T lymphocytes and also generate five- to six-fold fewer CTLs to the viral antigen. Similarly, mice lacking LMP7 demonstrate reduced cell surface levels of MHC class molecules (empty MHC molecules that do not carry antigenic peptides are unstable) and also demonstrate reduced T-cell response to the viral antigen HY (287). Thus the γ-IFN-induced alterations in the composition of the proteasomal subunits may increase the efficiency of antigen presentation and, consequently, of the immune surveillance.

Although the proteasome clearly is involved in cleavage at the C-terminal residue of antigenic peptides, the mechanism involved in specific cleavage at the N-terminal residue has yet to be identified. Recent evidence implicates the PA28 regulator of the proteasome in coordinated dual cleavages that lead to generation of the final antigenic peptides (289). The PA28 (designated also as the 11S regulator) strongly activates hydrolysis of peptides by the 20S proteasome in an ATP-independent manner, but it does not appear to be involved in degradation of intact proteins (290, 291). It is composed of two γ-IFN-inducible, approximately 27.5-kDa subunits, α and β. The chains form hexameric rings of approximately 200 kDa, composed of alternating subunits that associate loosely with the α endplates of the 20S proteasome and dissociate from it in low salt.

Overexpression of the PA28α (which is sufficient to stimulate the peptide-hydrolyzing activity) in mouse or human fibroblasts, along with the murine CMV pp89 protein, results in enhanced presentation to the appropriate CTL of an antigen derived from the viral protein. Enhanced presentation was observed also when an influenza nucleoprotein was expressed along with the α subunit. When purified PA28α was incubated along with purified 20S proteasome and a long peptide that contains the sequence of two antigens, one derived from the JAK1 protein of a mastocytoma cell line and the other derived from pp89, the protease trimmed the peptides on both sides to generate the authentic antigenic epitopes (289). However, because of the rarity of free peptides in the cytosol, the physiological role of PA28 is not clear.

Interestingly, it was reported that vaccination against Hsp70 derived from a sarcoma cell line (292) or from several autologous carcinomas, such as lung carcinoma, melanoma, and colon carcinoma, (292a) can render mice immune against the tumors. The Hsp associates with the antigenic peptide, and the function of the complex may be to carry the peptide from the site of its generation at the proteasome to the ER transport machinery. An Hsp-chaperoned peptide was shown to be channeled from the exogenous, MHC class II pathway to the endogenous, MHC class I pathway (293). A fraction of the proteasomes in the cell may exist as PA28/20S/19S heterotrimers. In this case, the proteasome will degrade intact proteins into large peptides, and the PA28 complex will trim the peptides to generate the antigenic epitopes.

Michalek et al (294) were the first to report that ubiquitinylation must precede processing of ovalbumin for presentation of the antigenic peptide SIINFEKL (amino acid residues 257–264) to the appropriate CTL. Using a mutant cell that harbors a thermolabile E1, the researchers showed that at the nonpermissive temperature, the peptide is not presented. Incubation at the high temperature, however, did not affect presentation of a peptide expressed in these cells from a minigene. This control experiment ruled out any defect in the transport or presentation machineries of the cell. Manipulation of genes that encode for antigenic proteins in a manner that renders the proteins more susceptible to ubiquitin-mediated degradation (such as conversion of the N-terminal amino acid residue into a "destabilizing" moiety) demonstrated that ubiquitin conjugation is a rate-limiting step in antigen presentation (295). Ben-Shahar et al (296) showed that production of SIINFEKL in a cell-free system from lymphocytes is mediated by the ubiquitin system: The process required ATP and ubiquitin, and could be reversibly inhibited by methylated ubiquitin. The in vitro experimental system may allow analysis of other components that may be involved in the process, such as molecular chaperones, which may be required to escort the generated peptides to the ER transport machinery, thus protecting them from cellular peptidases.

An interesting question involves recognition of protein antigens by the ubiquitin system. The Epstein-Barr virus (EBV) nuclear antigen 1 (EBNA1) persists in healthy virus carriers for life and is the only viral protein regularly detected in all EBV-associated malignancies. Unlike EBNAs 2–4, which are strong immunogens, EBNA1 is not processed and cannot elicit a CTL response. The persistence of EBNA1 contributes, most probably, to some of the pathologies caused by the virus. An interesting structural feature common to all EBNA1 proteins derived from different EBV strains is a relatively long Gly-Ala repeat of a variable length at the C-terminus domain of the molecule. Transfer of a strong antigenic epitope from EBNA4 (residues 416–424) to EBNA1 prevented its presentation, while its insertion in an EBNA1 deletion mutant that lacks the Gly-Ala repeat resulted in its presentation to the appropriate CTL. Similarly, insertion of the Gly-Ala repeat downstream to the 416–424 epitope in EBNA4 inhibited CTL recognition of the EBNA4 chimeric protein (297). Thus, the Gly-Ala repeat constitutes a *cis*-acting element that inhibits antigen processing and subsequent presentation of the resulting antigenic epitopes.

A more recent study carried out in a cell-free system showed that EBNA4 is degraded in an ATP-, ubiquitin-, and proteasome-dependent manner, whereas EBNA1 is resistant to proteolysis. However, EBNA1 is degraded by the ubiquitin cell-free system following deletion of the Gly-Ala repeat. Transfer of the signal to EBNA4 prevented, as expected, its degradation by the ubiquitin system (184). A short Gly-Ala repeat (38 amino acid residues) as well as a short Pro-Ala repeat have similar effects. Interestingly, the presence of the Gly-Ala repeat does not prevent ubiquitin conjugation to Gly-Ala-containing EBNA4. This finding suggests that the Gly-Ala repeat, like the Gly-rich region in p105 (see above), interferes with processing of the protein by the 26S proteasome. Since a Gly-rich region as well as Gly-Ala or Gly-Pro repeats of various lengths have similar inhibitory effects on processing, it appears that a formation of domains composed of small uncharged/hydrophobic residues prevent entry into the proteasome, thus inhibiting degradation of an already ubiquitin-tagged protein.

CONCLUDING REMARKS

Spectacular progress has been achieved in the past few years in our understanding of the important functions of selective, ubiquitin-mediated protein degradation in a variety of cellular processes. Ubiquitin-mediated protein degradation may be comparable to protein phosphorylation in the variety of its regulatory functions. This seemingly wasteful mechanism of using disposable protein regulators may be essential to ensure irreversibility of temporally controlled processes, such as cell cycle or development. The rapid degradation

of protein regulators is especially important when the regulator should act for a short period of time (e.g., cyclins or some transcriptional regulators) or when a process is initiated by the degradation of an inhibitor (e.g. degradation of Ckis or IκBs). Further examples of ubiquitin-mediated regulatory processes will likely be discovered in the near future. For instance, it remains to be seen whether the rapid degradation of components of the circadian clock (298) is carried out by the ubiquitin system, and what controls the precise timing of its degradation.

We still know very little about the ubiquitin ligases (E3 enzymes), which are responsible mainly for the selectivity and regulation of ubiquitin-mediated protein degradation. As discussed above, this is partly because of the divergence of different families of E3 enzymes and should be tackled by a combination of biochemical and molecular genetic approaches. For example, work with cell-free systems is needed to determine whether Mdm2, which is required for the degradation of p53 (202, 203), or the APC protein required for the degradation of β-catenin (206, 208) are parts of E3 enzyme complexes that act in the ubiquitinylation of the respective proteins. Of equal importance is the identification of signals in proteins that are recognized by the different E3 enzymes. Significant progress has been made in this respect, such as the identification of the role of phosphorylation in the degradation of many proteins, but this is just the tip of the iceberg. Different phosphorylated degradation signals are likely recognized by different E3 enzymes, and many other degradation signals probably exist. The elucidation of other types of interplay between protein phosphorylation and protein degradation, as illustrated by the regulation of cyclosome activity in the cell cycle, are major challenges for the future.

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