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Ubiquitination and deubiquitination: Targeting of proteins for degradation by the proteasome

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The post-translational modification of proteins by covalent attachment of ubiquitin targets these proteins for degradation by the proteasome. An astounding number of proteins are involved in ubiquitination and deubiquitination of proteins. The pathways are combinatorial, and selectivity of proteolysis will depend strongly on the exact combination of ubiquitinating and deubiquitinating enzymes present at any time. In addition to temporal control, it is likely that these modifications are also regulated spatially. In this review, we discuss the regulation of ubiquitination by enzymes of this pathway and highlight some of the outstanding problems in understanding this regulation.

Key words: deubiquitinating enzymes / proteolysis / regulation / ubiquitin / ubiquitin ligase

Introduction

ONE OF THE MAJOR QUESTIONS OF cellular regulation and development centers on how the cell selects proteins to be degraded at an appropriate time and place.[1–6] Since the discovery of the cyclins, it has been apparent that this process is vital and highly regulated. Over the past 20 years, it has become obvious that a major mechanism of selective protein degradation involves the post-translational modification of proteins by the small protein ubiquitin, and delivery of these modified proteins to the proteasome.[3,7] This system is far more complicated than previously appreciated and is estimated to involve over 5% of the genes in yeast.

The process of selective proteolysis can be thought of as consisting of three steps: identification of the protein to be degraded; marking of that protein for degradation by attachment of ubiquitin (ubiquitination); and delivering it to the proteasome, a multienzyme protease complex that will degrade it and recycle ubiquitin. In many cases, identification of a protein for ubiquitination involves a genetically encoded ubiquitination signal and/or a prior modification such as phosphorylation, or damage to the protein.[8] Thus, ubiquitination can best be thought of as a signal for localization of the protein to the proteasome and the regulation of the ubiquitination state of a particular protein will influence the half-life of that protein.

The ubiquitin domain is a targeting signal

There are a large number of ubiquitin-like proteins, and ubiquitin domains are also found in larger proteins. While it is beyond the scope of this review, a brief discussion of these proteins will help the reader to appreciate that the ubiquitin domain is a versatile targeting signal that has been used many times in evolution. Ubiquitin is a 76 amino acid protein universally distributed among eukaryotes and highly conserved. Only three amino acids differ between yeast and human ubiquitin. The ubiquitin system is the prototype for a newly recognized family of pathways that result in the covalent modification of target proteins (Table 1). These systems use different ubiquitin-like proteins, different enzymes, and act on different substrates, but share a common chemistry.[9–13] The number and types of processes affected is similarly diverse. Current dogma would suggest that attachment of a single ubiquitin domain to a target protein serves to modulate activity or location, while attachment of multiple ubiquitins

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as a K48-linked polyubiquitin chain (see below) results in targeting to the proteasome and subsequent proteolysis of the target protein.

Ubiquitination signals

Ubiquitination signals on target proteins can be genetically programmed, or can be acquired by phosphorylation, by binding to an adapter protein, or by protein damage due to fragmentation, oxidation, or aging.[3, 8, 14]

Genetically programmed ubiquitination signals

Many proteins have short sequences of amino acids that are necessary and sufficient to direct their ubiquitination. The first of these was discovered by Varshavsky's group[15] and consists of a short N-terminal peptide derived from the beta-galactosidase cloning vectors. This was referred to as the N-end rule degron, because the rate of ubiquitination depends strongly on the identity of the N-terminal residue. Since then, a number of other sequences have been identified although there is no obvious consensus sequence other than the presence of significant hydrophobicity.[16] This probably reflects the fact that a large number of ubiquitin ligases are involved in ubiquitinating proteins carrying such signals (see below). In many cases, the exposure or accessibility of these sequences is modulated by binding to ligands, or by additional covalent modifications.

Phosphorylation regulates ubiquitination

In the early 1990s, phosphorylation of cell surface receptors in response to ligand engagement was shown to be correlated with receptor ubiquitination and degradation.[14, 17, 18] Subsequently, the ubiquitination of a large number of cellular regulators have been shown to be regulated by phosphorylation. These include: cyclins and cyclin kinase inhibitors; other checkpoint regulators such as cdc6p (replication) Swe1p (budding); signal transduction components (such as SMAD's, TGF-β, cb1) and numerous cell surface receptors and transporters, and proteins regulating transcription such as IκBα, β-catenin, p53, Jun, and even RNA polymerase.[8, 19] In addition, the activity of ubiquitin ligases can be modulated by phosphorylation.[20] Thus, the pathways of proteolysis are integrated with other signaling pathways and cellular regulation events. The recognition of phosphorylated proteins by the ubiquitin pathway is probably best explained by the numerous F-box proteins[21] that specifically bind to phosphoproteins and are integral components of many ubiquitin ligase complexes (see below).

Damage to proteins targets them for ubiquitination

The ubiquitin system was first identified as a pathway for the degradation of damaged proteins and that is the other major role it plays. Proteins damaged by oxidation or mutation or that misfold or mislocalize are good substrates for this system. Little is known about how these are recognized, although recent models invoke interaction with chaperones and the cytoskeleton. Abnormal proteins in the cytoplasm are bound to the microtubule network and localized to the centrosome along with many of the enzymes of the pathway.[22, 23] Misfolded proteins in the ER are shuttled back to the cytoplasm by retrograde transport and ubiquitinated and degraded there.[24]

Ubiquitinating enzymes

Ubiquitin is activated at its C-terminus by adenylylation and subsequent rearrangement to form an intermolecular thiol ester with the E1-activating enzyme. Most organisms contain one or two such ubiquitin-activating enzymes. The activated ubiquitin is then passed on to a thiol group on one of a dozen or so E2 ubiquitin-conjugating enzymes. Finally, these activated E2s are bound by one of several dozen E3 ubiquitin ligases and transferred to the target protein.[3] Figure 1 summarizes this complexity. Many parallel pathways of protein ubiquitination are possible, depending on which E2 and E3 are utilized. The ubiquitinated proteins that result are then all degraded by the proteasome, or perhaps a few different types of proteasomes. This metabolic logic makes it clear that the regulation of which proteins become ubiquitinated must reside largely at the level of E2 and E3.

The conjugation of ubiquitin to target proteins is catalysed by a complex containing an activated conjugating enzyme (a thiol ester between ubiquitin and a conjugating enzyme), and an ubiquitin protein ligase. There are five types of ligases currently recognized.[3, 5] The first of these discovered, the E3α ligase, recognizes the N-terminal residue on the substrate and is responsible for the substrate specificity of the N-end rule pathway.[15] A second class of ligases contains a signature domain referred to as a HECT domain

Table 1. Conjugation of ubiquitin-like proteins is a targeting signal. Yeast proteins are indicated with the suffix p. The similarity between Apg12p and ubiquitin is slight, although it is included here because of the similar enzymes and chemistry used. See the referenced reviews[2–5, 8–13] and the text for details

Modifier	Activating enzyme(E1)	Conjugating enzyme(E2)	Processes regulated	Substrates	Fates
UBIQUITIN	Uba1p	Ubc1–8p,	DNA repair		Regulation
		Ubc10–11p,	Peroxisome biogenesis		”
		Ubc13–16p	Mito. protein location		”
			DNA silencing		”
			DNA replication		”
			Tumor suppression	p53	Proteolysis
			Transcription	1κBα, HIF-1, etc.	”
			Stress/protein damage	Damaged proteins	”
			Receptor internalization	Surface receptors	”
			Protein processing	NFκB	”
NEDD8/Rub1p	U1a1p, Uba3p	Ubc12p	Ubiquitination	Cdc53p	Ligase localization?
UCRP			Interferon-induced		Cytoskeletal localization
SUMO-1/Smt3p	Aos1p, Uba2p	Ubc9p	Nuclear transport	RanGAP	Nuclear localization
			Nuclear dot formation	PML, Sp100, HIPK2	Subnuclear localization
			Cytokinesis	Cdc3p	Bud Neck localization
			Transcription	IκBβ, p53, HIPK2	Inhibition of ub’n
Apg12p	Apg7p	Apg10p?	Autophagic vesicle	Apg5p	Membrane localization

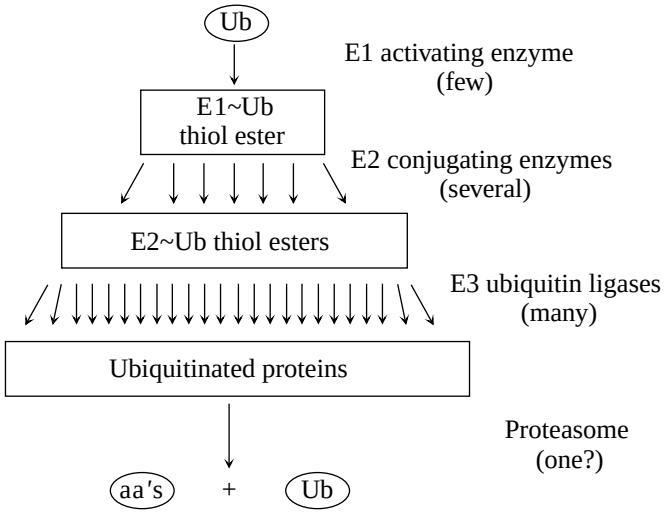

Figure 1. Combinatorial nature of ubiquitination. The pathways diverge at the level of the E2 and E3 enzymes responsible for ubiquitination of individual substrate proteins, and then converge again at the level of the proteasome. Details are given in the text.

(homologous to E6AP C-terminus). The prototype of this family is the E6AP mammalian protein involved in the degradation of the tumor suppressor p53 in cells infected with the human papilloma virus. E6AP binds to a complex between p53 and the viral E6 protein (an adapter protein), resulting in the polyubiquitination of p53. Mutations in E6AP have been suggested to cause Angelman’s Syndrome, a profound mental retardation.[25] The third type of ubiquitin protein ligase is a multienzyme complex referred to as the anaphase-promoting complex or cyclosome (APC/cyclosome) because its activity is required to ubiquitinate cyclins and other substrates degraded at the metaphase–anaphase transition.[6, 20, 26] Substrate recognition is thought to require one of several adapter proteins containing WD40 repeats. A third multienzyme complex catalysing ubiquitination, the SCF ligase, consists of a Skp1, a cullin, and an F

box protein.[21,27,28] Numerous F-box proteins are known and this component is thought to recognize the protein substrates. Finally, the CBC ligases are formed by association of a cullin, and the elongin B/C complex. Interestingly, this complex can bind to the von Hipple–Lindau tumor suppressor protein (VHL) to modulate the half-life of anoxia-regulated mRNAs. It is thought that the Cul2/elongin BC/VHL complex is the ligase responsible for the ubiquitination and degradation of a specific transcription factor, HIF1α, under normoxic conditions.[29]

Deubiquitinating enzymes

The conjugation of ubiquitin-like proteins is a reversible process. There are at least 19 proteins in yeast that are members of three protein families that catalyse the hydrolysis of the peptide bond at G76 of the ubiquitin domain.[30,31] Many more are known to exist in higher eukaryotes (see below). Some of the putative roles for deubiquitinating enzymes in the ubiquitin system are shown (Figure 2).

There are two classes of specific proteases acting on polymeric ubiquitin and cleaving at the C-terminal glycine of ubiquitin. These have been called deubiquitinating enzymes (DUB), and consist of the ubiquitin C-terminal hydrolases (UCH) and the ubiquitin-specific processing proteases (UBP). In general, the UCH isozymes are thought to be involved in processing ubiquitin-fusion proteins and are more active on ubiquitin extended by small peptides or larger substrates with a flexible peptide linking the C-terminal domain. UBP enzymes, on the other hand, are thought to be responsible for removing ubiquitin from larger proteins and disassembling the polyubiquitin chains. Less is known about the processing of ubiquitin-like proteins, but two examples of deubiquitinating enzymes acting on SUMO1 have recently been identified and Hochstrasser has reserved the name ULP (ubiquitin-like protease) for this enzyme family.[32]

UCH enzymes have important roles in development and neural function

Ubiquitin C-terminal hydrolases are papain-like thiol proteases with a 230 amino acid core catalytic domain and specificity for cleavage of ubiquitin derivatives with small or disordered C-terminal domains.[31] A number of mutations in UCH family members appear to be associated with disease. An I93M mutation in human UCH-L1, a neuronal-specific isoform also known as PGP9.5, may be associated with the development of Parkinson's disease in a small minority of the cases.[33] Recently, deletion of exons 7 and 8 containing catalytically important residues has been shown to be the cause of gracile axonal distrophy in the mouse.[34] This disease is characterized by sensory and motor ataxia and exhibits a 'dying-back' type axonal degeneration and formation of spheroid bodies in nerve terminals. Finally, it has been shown that a similar enzyme is induced upon long-term facilitation in *Aplysia*. The evidence suggests that the induction of this UCH is necessary for long-term, but not short-term, facilitation.[35]

A second UCH may be associated with disturbances of growth regulation, including lung cancers. BAP1, a related UCH family member that we have recently characterized, differs from the other family members in having a 500 amino acid C-terminal domain that interacts with the ring-finger domain of BRCA1, the breast cancer tumor suppressor. Three mutations have been noted, all in concert with a loss of heterozygosity or mutation in the other allele.[36] We speculate that the C-terminal extension may be involved in recruiting the UCH domain to its site of action.

UBP enzymes regulate signal transduction, growth, and development

A second family of DUB enzymes is the UBPs. The core catalytic domain of these thiol proteases is about 350 amino acids long, and there are sixteen UBPs in yeast. They vary from 50 to 250 kDa with a variety of N-terminal extensions, an occasional C-terminal extension, and insertions in the catalytic domain. These extensions are thought to contribute to substrate specificity and/or localization. No structures of this family are known.

The first of these family members to be genetically and biochemically characterized was Ubp4 from yeast. Mutants in this protein are defective in the degradation of the MATα2 mating factor in yeast. These cells accumulate polyubiquitin chains with peptide remnants still attached, show impaired proteolysis, defects in DNA replication, and lower steady-state values of free ubiquitin. It is likely that this enzyme is responsible for removing the polyubiquitin chain from the residual peptide that remains after proteasomal degradation of a polyubiquitinated substrate.[37]

Cytokine-induced signaling events induce a family of mammalian deubiquitinating enzymes involved in the regulation of growth. These DUBs were shown

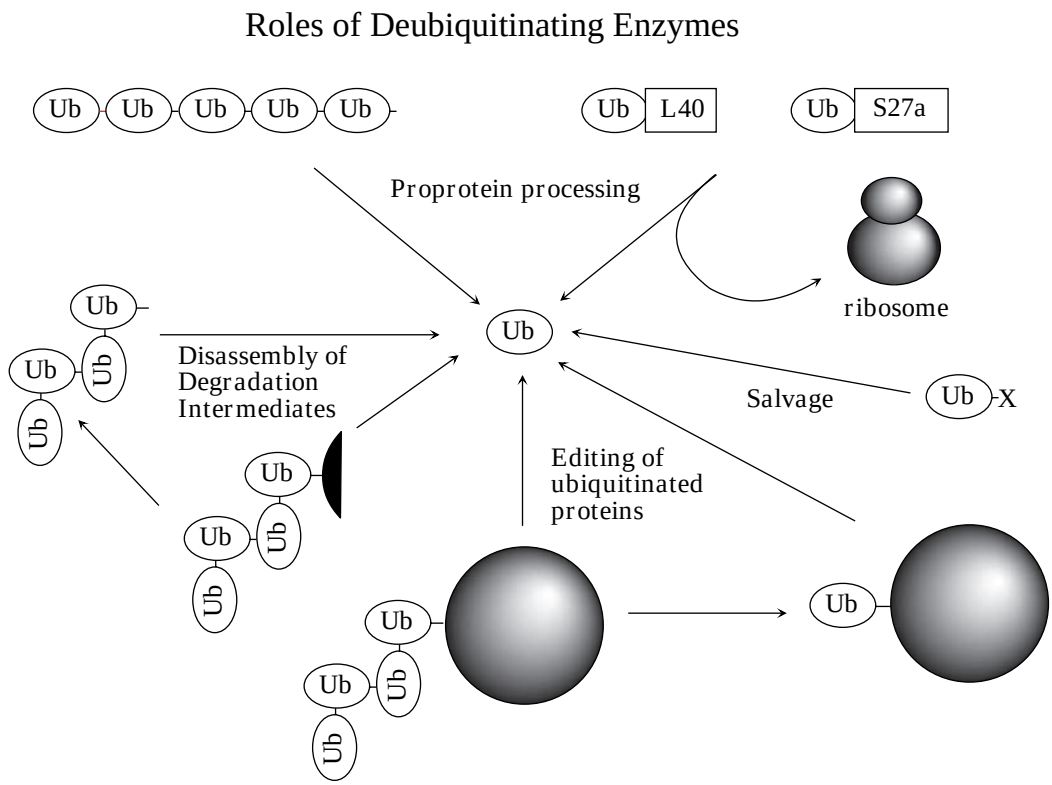

Figure 2. Possible roles for DUB enzymes. Ubiquitin and several ubiquitin-like proteins are synthesized as fusion proteins and must be processed to the mature domain by cleavage at the C-terminal glycine. Second, the C-terminus of ubiquitin must be regenerated after degradation of the attached protein and any adventitious reaction of ubiquitin thiol esters with cellular amines and thiols must be reversed by processing at the C-terminus. Third, deubiquitinating enzymes could act as 'proofreading' enzymes to reverse ubiquitination of the 'wrong' protein, or even the 'right' protein at an inappropriate time or place. Finally, the polyubiquitin chain must be disassembled after use to replenish the ubiquitin pool and prevent accumulation of free chains that could act as competitive inhibitors of the binding of ubiquitinated proteins to the proteasome.

to be immediate early gene products produced upon cytokine stimulation, with each mechanistically distinct interleukin inducing a different isozyme of this deubiquitinating enzyme. The JAK2 pathway is involved in transducing the signal and overexpression of these enzymes results in suppression of growth (see Reference 30 and references therein).

A newly identified DUB (Ubp-M) is phosphorylated at the onset of mitosis and dephosphorylated during the metaphase/anaphase transition.[38] Mutant forms, lacking an active-site cysteine, associate closely with mitotic chromosomes during all stages of cell division and remain within the nucleus during the post-mitotic period. Ubp-M may deubiquitinate one or more critical proteins that are involved in the condensation of mitotic chromosomes.

Several other processes are modulated by DUBs: receptor internalization has been shown to require UBP4; transcriptional silencing in yeast is inhibited by UBP3; and position effect variegation in *Drosophila* is inhibited by a UBP. UBP3 has been shown to bind to Sir4, a component of the telomeric silencing complex (see the reviews cited above). This clearly suggests that DUBs are targeted to specific sites in the cell and points to a role for deubiquitinating enzymes in cellular compartments other than the cytoplasm.

DUBs have also been shown to have profound effects on development. The FAF gene in *Drosophila* codes for a DUB that is involved in development of the photoreceptors in the eye,[39] modulating the activity of the Ras/receptor tyrosine kinase pathway. A similar enzyme exists in mouse where it is found to bind at the cell membrane at cell–cell contacts, and to bind to AF-6, a downstream target of Ras that is degraded by the ubiquitin-dependent pathway and thought to be involved in adhesion-dependent

regulation processes.[40] In *Dictyostelium*, insertional mutagenesis of the isopeptidase T gene results in defects in aggregation.[41] Mutants accumulate free polyubiquitin chains that are thought to bind to the proteasome interfering with the degradation of a regulator of developmental checkpoints.

Ubiquitin receptors and binding proteins

While the attachment of K48-linked polyubiquitin chains targets proteins for degradation, polyubiquitin chains can also be synthesized with linkages between the C-terminus of ubiquitin and K6, K11, K29, and K63 on the adjacent ubiquitin. The precise role of these linkages is unknown, although there is genetic evidence that K29-linkages are necessary for degradation of ubiquitin-fusion proteins and K63 linkages are vital in regulating DNA repair and ubiquitination of cell surface receptors, but not required for growth or degradation of short-lived proteins.[42] Understanding the variety of targeting functions conferred by ubiquitination demands that we identify and characterize putative binding proteins and receptors. The binding of ubiquitin and ubiquitin chains has been characterized in only four cases; two DUBs,[43,44] the proteasome subunit S5a,[45] and a signaling adapter P62.[23]

The binding site for ubiquitin on UCHs has been identified and described using NMR and X-ray crystallography.[43,46,47] The basic face of ubiquitin interacts with acidic residues on human and yeast UCHs to form a salt-sensitive enzyme–product complex. Another DUB, isopeptidase T, binds polyubiquitin chains *via* hydrophobic interactions with up to four ubiquitin molecules, although no structures are known. S5a, a subunit of the 19S regulator of the proteasome binds to polyubiquitin chains on Western blots. Little information is available about its binding in solution and this subunit is dispensable in yeast suggesting that there are other polyubiquitin binding sites on the proteasome. Finally, human P62, an adapter that binds to signaling kinases in a phosphorylation-independent manner has been shown to interact in a two-hybrid screen and to bind to immobilized ubiquitin. In the mouse, this protein is known as STAP/A170 and is induced by oxidative stress. It has been found to be associated with damaged proteins found in intracellular hyalline bodies and associated with the centrosome in a structure called the aggresome or the sequestosome.[23,48] This is a strong candidate for the targeting receptor that is responsible for binding ubiquitinated proteins to the microtubules and recruiting them to the centrosome. This localization may sequester these proteins in a locale rich in proteasomes, ubiquitinating enzymes, and other components of the degradative pathway.

No consensus sequence for a ubiquitin-binding protein is known. The UBA domain is found in several proteins of the ubiquitin pathway and has been annotated as a ubiquitin-binding domain,[23] but there is no direct evidence of its involvement in binding ubiquitin. Isopeptidase T and the p62 chain-binding protein contain it, while UCH isozymes and S5a do not. In light of the numerous pathways that bind ubiquitin domains, it may be that receptors and binding proteins will prove to be a diverse group with little in common.

Summary

Over the past 20 years, it has become apparent that the ubiquitin domain has been utilized as a general targeting domain that is post-translationally added to proteins and that participates in a number of important pathways of physiological regulation. In proteolysis, we know that K48-linked polyubiquitin chains are bound by the proteasome, but a number of questions remain unanswered. Why are there so many ubiquitinating enzymes if prior modifications such as phosphorylation or damage are triggering events? Do DUBs show substrate specificity, perhaps by regulating the levels of ubiquitination of specific subsets of proteins? What are the binding sites for polyubiquitin chains on the microtubules and on the proteasome itself? What is the role of K29- and/or K63-linked polyubiquitin chains in the cell? There are clearly enough questions for the next 20 years.

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