Critical Reviews in Biochemistry and Molecular Biology, 33(5):337–352 (1998)

Deubiquitinating Enzymes: A New Class of Biological Regulators

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ABSTRACT: Protein ubiquitination controls many intracellular processes, including cell cycle progression, transcriptional activation, and signal transduction. Like protein phosphorylation, protein ubiquitination is dynamic, involving enzymes that add ubiquitin (ubiquitin conjugating enzymes) and enzymes that remove ubiquitin (deubiquitinating enzymes). Considerable progress has been made in the understanding of ubiquitin conjugation and its role in regulating protein degradation. Recent studies have demonstrated that regulation also occurs at the level of deubiquitination. Deubiquitinating enzymes are cysteine proteases that specifically cleave ubiquitin from ubiquitin-conjugated protein substrates. Genome sequencing projects have identified many candidate deubiquitinating enzymes, making them the largest family of enzymes in the ubiquitin system. Deubiquitinating enzymes have significant sequence diversity and therefore may have a broad range of substrate specificities. Here we explore the structural and biochemical properties of deubiquitinating enzymes and their emerging roles as cellular switches.

I. INTRODUCTION

A. Protein Ubiquitination as a Mechanism of Biological Regulation

Protein ubiquitination controls such diverse biological processes as cell cycle progression,[14,26] apoptosis,[15,42,43] and growth factor-mediated signal transduction.[5,45,46] For some protein substrates, ubiquitination leads to protein degradation by the 26S proteasomal complex. For other protein substrates, ubiquitination results in a reversible post-translational modification, regulating cellular targeting and enzymatic activity (Table 1).

It is instructive to compare and contrast protein ubiquitination with protein phosphorylation. Protein phosphorylation is controlled by the counterbalancing action of protein kinases and protein phosphatases.[48] Originally, kinases were viewed as the key regulators of protein phosphorylation *in vivo*, while a small number of phosphatases were thought to play largely housekeeping roles. The importance of phosphatases emerged after a few critical regulatory phosphatases were identified. Genome sequence information has now identified a vast array of phosphatases whose stuctural diversity suggests a diversity of substrate specificities.[8,27] In the current view, either phosphatases or kinases can perform regulatory functions. The predominant mechanism (phosphorylation vs. dephosphorylation) is determined by the architecture of each specific regulatory circuit.

TABLE 1
Cellular Functions of Protein Ubiquitination

	Ref.
1. Ubiquitin-mediated proteolysis targeting poly-ubiquitinated protein to the proteasome	Ciechanover, 1994 Finley, 1991 Hershko, 1988
2. Targeting to the vacuole	Hicke, 1996
3. Activation of enzyme activity — Ikβ kinase activation	Chen, 1996
4. Activation of cytokine receptor-mediated signal transduction	Strous, 1996 Strous, 1997 Kim, 1996

Like phosphorylation, protein ubiquitination is controlled by the coordinate action of multiple ubiquitin conjugating enzymes and deubiquitinating enzymes. There is a remarkable diversity of structurally distinct deubiquitinating enzymes. In this review we discuss the role of deubiquitinating enzymes at multiple levels of the proteasome-degradation pathway. In addition, we discuss potential regulatory roles for deubiquitinating enzymes outside the proteasome pathway.

II. DEUBIQUITINATING ENZYMES

A. Multiple Cellular Functions of Deubiquitinating Enzymes in the Proteasome Pathway

Deubiquitinating enzymes are cysteine proteases that specifically cleave ubiquitin conjugates at the ubiquitin carboxy terminus. The ubiquitin moiety may be cleaved from ubiquitin esters, ubiquitin thiol esters, or ubiquitin amides. Deubiquitinating enzymes act at multiple levels in the ubiquitin pathway (Figure 1). These enzymes are responsible for the processing of linear polyubiquitin chains to generate free ubiquitin from precursor fusion proteins (level 1). Furthermore, these enzymes impact on free ubiquitin pools by recycling ubiquitin from branched-chain polyubiquitin (Level 2). For instance, a critical enzyme that works at this level is isopeptidase T/ubp14 (discussed below). Deubiquitinating enzymes also remove ubiquitin from Ub-conjugated target protein, thereby regulating the localization or the activity of the target (Level 3). Another biochemical role of deubiquitinating enzymes is to remove ubiquitin from a ubiquitinated target protein and to rescue the protein from degradation by the 26S proteasome (Level 4). This could occur either before or after the ubiquitinated substrate interacts with the proteasome. The substrate may be rescued either by removing the branched-chain ubiquitins en masse or by trimming the distal ubiquitin subunits sequentially. Finally, deubiquitinating enzymes play an important role in clearing the proteasome of peptide remnants conjugated to ubiquitin chains. Removal of ubiquitin from these protein remnants may "unclog" the barrel-shaped proteasome and thereby maintain or increase proteasomal activity (Level 5). At all five levels, the deubiquitinating enzymes remove intact ubiquitin and, to a greater or lesser degree, impact on free intracellular ubiquitin pools.

B. Characteristic Structural Features of Deubiquitinating Enzymes

There are two major families of deubiquitinating enzymes, the ubp family (ubiquitin processing proteases)[3,39,47] and the uch family (ubiquitin carboxy terminal hydrolase).[29,52] The ubp family and the uch family have also been referred to as type 1 Uch and type 2 Uch families, respectively.[50] In general, more is known about the biological function of ubps.

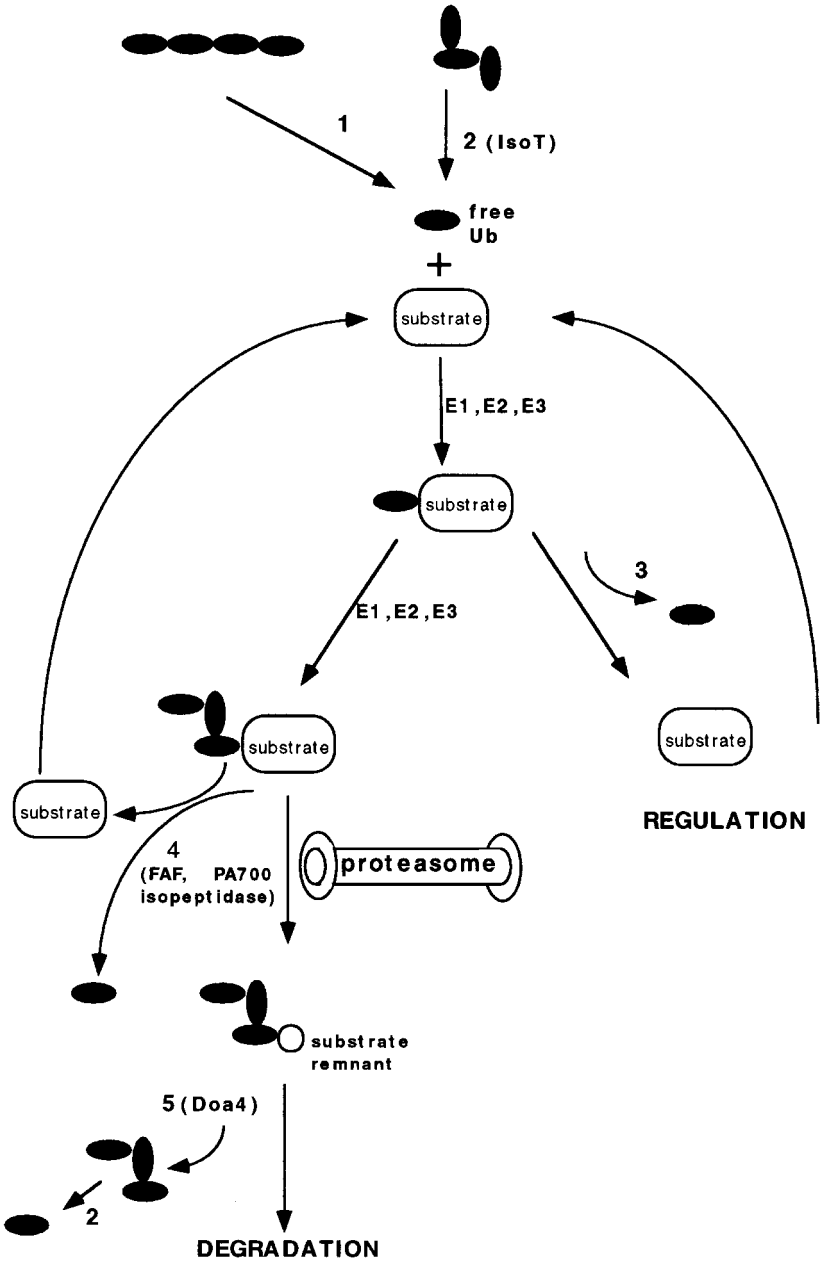

FIGURE 1. Multiple cellular functions of deubiquitinating enzymes. Deubiquitinating enzymes act at several different levels in the regulation of protein ubiquitination. In (1), a deubiquitinating enzyme degrades a linear polyubiquitin chain into monomeric ubiquitin molecules (76 amino acids per monomer). In (2), a deubiquitinating enzyme, such as isopeptidase T, degrades a branched multiubiquitin chain into monomeric ubiquitin molecules. Following ubiquitination, some proteins are targeted for degradation via the ubiquitin-mediated proteolytic (proteasomal) pathway. Other ubiquitinated proteins remain stable, but have regulated cellular localization or activity, depending on their state of ubiquitination. In (3), a deubiquitinating enzyme removes ubiquitin from a Ub-conjugated target protein and thereby regulates the localization or activity of the substrate. In (4), a deubiquitinating enzyme, such as FAF or PA700 isopeptidase, removes polyubiquitin from a ubiquitinated target protein and thereby rescues the protein from degradation by the 26S proteasome. In (5), a deubiquitinating enzyme, such as Doa4, removes polyubiquitin from proteasome degradation products. Removal of ubiquitin from these protein remnants may increase net substrate degradation. At all five levels the deubiquitinating enzymes remove ubiquitin and thereby regulate the cellular pool of free monomeric ubiquitin.

C. Ubp Family

Ubps were originally expression cloned by their ability to deubiquitinate a ubiquitin-B-galactosidase fusion substrate.[3,47] Ubps greatly vary in length and structural complexity.[39,53] While there is little amino acid sequence similarity within their coding region, sequence comparison reveals some consistent features. Like many other cysteine proteases, ubps contain a "catalytic triad" of amino acids, including a cysteine, aspartic acid, and histidine residue (Figure 2). For ubps, an essential role in catalysis of the conserved cysteine and histidine residues has been established,[23,39] while the importance of the aspartic acid residue remains untested. Short sequences surrounding the cysteine residue (Cys domain) and histidine residue (His domain) are highly conserved among all ubps.[39]

In total, there are six conserved regions of ubps[53] (Figure 2). One region surrounds the conserved cysteine residue (DHI, for Deubiquitinating enzyme Homology domain 1), one surrounds the aspartic acid residue (DHII), one surrounds the histidine residue (DHV), and three additional regions have unknown function. One or more of these other regions may provide a ubiquitin binding site(s). Although not tested yet, it seems likely that these six regions will be required for the catalytic activity of the ubp. These core sequences contain approximately 300 to 500 amino acids and span the sequence from the active site cysteine in DHI domain to the carboxy terminal end of DHVI domain. Together, these six domains provide a molecular signature for the ubp family, allowing rapid identification of new ubps in genome databases. Although the crystal structure of a ubp is not yet available, presumably these six domains will turn out to have a highly conserved structure.

Among ubp family members, little primary amino acid sequence homology exists outside of the core catalytic domain.[2] Ubps vary greatly in size, ranging from 526 amino acids for *DUB1* to 2691 amino acids for the FAF protein (Figure 3). Outside of the core catalytic domain, ubps have N-terminal or C-terminal extensions. For instance, ubp14, ubp3, and Doa4 have N-terminal extensions. By contrast, *DUB-1* has a C-terminal extension. These additional sequences of ubps presumably have a role in determining substrate specificity or cellular localization.

A sequence comparison of several ubp family members also reveals subfamilies. One subfamily includes the *DUB* enzymes, which are transcriptionally induced in response to cytokines.[56–58] One short peptide region within the C-terminal extension of *DUB* family members shows remarkable sequence diversity. This "hypervariable region" may have a role in the recognition of specific substrates.[57,58] A tandem repeat of *DUB* genes maps to a region of murine chromosome 7,[57,58] suggesting that the *DUB* subfamily arose by tandem duplication of an ancestral *DUB* gene.

D. Uch Family

Distinct from the ubp family is the ubiquitin carboxy-terminal hydrolase (uch) family of enzymes.[29,31,51,52,54] Uchs were originally purified on the basis of their ability to bind ubiquitin affinity columns.[54] Like ubps, uchs are cysteine proteases containing an active site cysteine, aspartate, and histidine residue. Uchs do not, however, contain the six characteristic homology domains of the ubps.[29] The uch family includes a group of small, closely related proteases consisting of three human isozymes[51,52] with close homologues in *S. cerevisiae*,[30] *Drosophila*,[55] and Aplysia.[19] Recent data confirm the importance of cysteine and histidine residues in catalytic activity. Uchs have also been shown to have a single specific ubiquitin binding

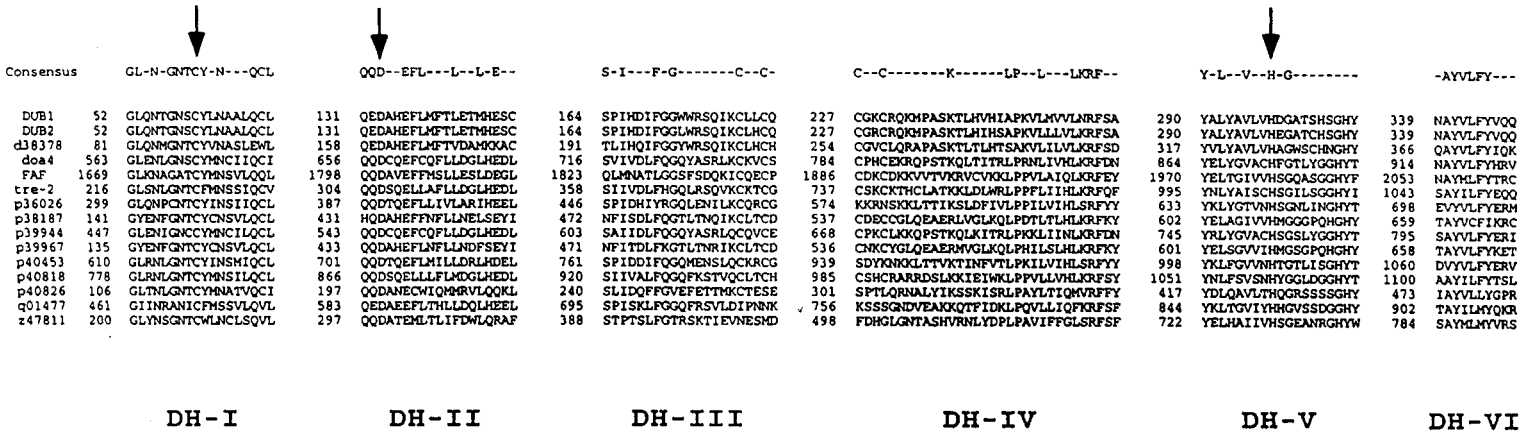

FIGURE 2. Conserved structural features of the ubp families of deubiquitinating enzymes. Amino acid alignment of several members of the ubp superfamily, using Blockmaker server (URL http://www.blocks.fhc.org). Six *DUB* homology domains (DHI through DHVI) are shown.[53] The DHI domain contains a conserved Cysteine residue (C). The DHII domain contains a conserved aspartic acid residue (D). The DHV domain contains a conserved histidine residue (H). These Cys, Asp, and His residues comprise the "catalytic triad" of the active site of the enzyme. The other DH domains have unknown function, but presumably contribute to the formation of the active site. One domain may also play a role in ubiquitin binding. Sequence names and accession numbers are as follows: TRE2_HUMAN, SwissProt:p35125; UBPJ_YEAST, SwissProt:p39538; DOA4_YEAST, SwissProt:p25037; UNPH_HUMAN, GenPept:u20657; UBP_MOUSE, SwissProt:p35123; UBPCE_CAEEL, GenPept:z47811; UBPE_YEAST, SwissProt:p39967;UBPB_YEAST,SwissProt:p38187; UBP2_YEAST,SwissProt:q01476;UBPC_YEAST,SwissProt:p38237; UBP1_YEAST, SwissProt:p40453;UBP3_YEAST,SwissProt:q01477;UBPK_YEAST,SwissProt:p36026; FAF_DROS, PIR:a49132; TGT_RABIT, SwissProt:p40826; UBPX_CAEEL, SwissProt:p34547; UBP5_YEAST, SwissProt:p39944; UBPX_HUMAN, SwissProt: p40818; HSAN_HUMAN, EMBL:d38378.

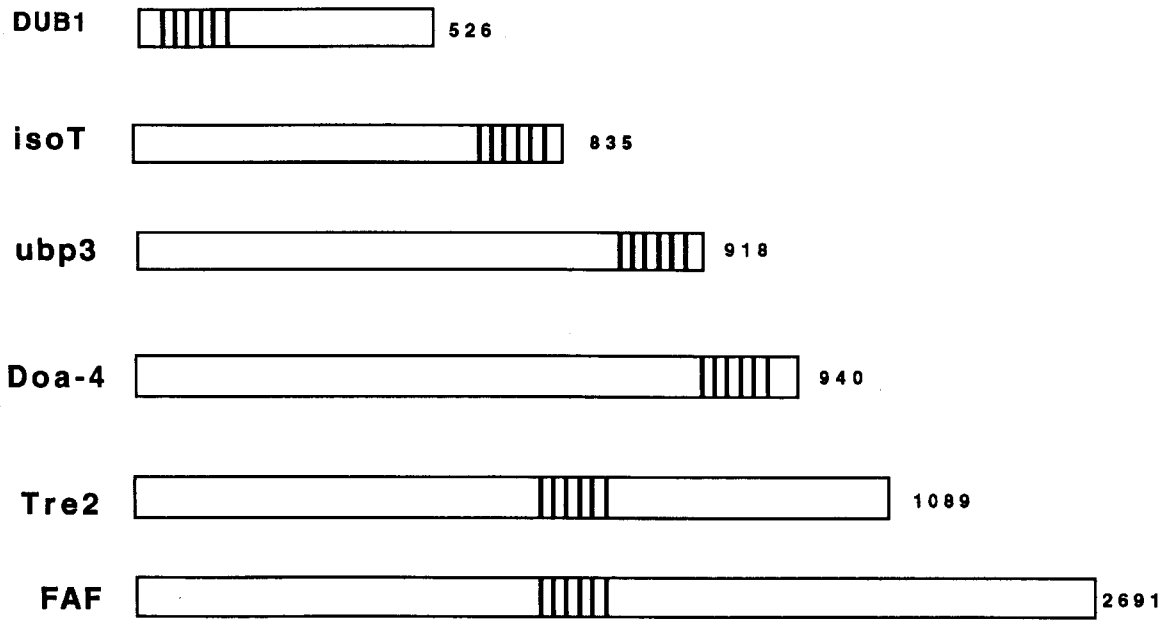

FIGURE 3. Diversity within the deubiquitinating enzyme (ubp) superfamily. Schematic representation of six members of the ubp superfamily described in the text. The six ubp homology domains comprising the core catalytic domain are shown. Regions outside of the core catalytic domain may determine unique biochemical or biological functions of each enzyme, as described in the text. *DUB-1* contains a lysine-rich region and a hypervariable region.

site, unlike the ubp family, whose members may have multiple ubiquitin binding sites.[44]

The crystal structure of a human Uch, Uch-L3, suggests interesting mechanistic features of this class of enzyme.[24] There are significant similarities to the papain-like cysteine proteases. As expected, the active site contains the catalytic triad of cysteine, histidine, and aspartic acid residues. Interestingly, the catalytic site is masked by three different segments of the enzyme. By analogy with inhibitor complexes of papain-like enzymes, it is proposed that ubiquitin binding to Uch-L3 induces a conformational change that allows access to the active site. This proposed conformational change may provide a mechanism to prevent nonspecific hydrolysis.

In terms of substrate specificity, uch family members preferentially cleave ubiquitin from small adducts such as peptides and amino acids. Ubps, in contrast, may be specific for larger leaving groups, such as proteins. The uch and ubp families of thiol proteases are distinct (Figure 2) and share little sequence homology with each other or with other cysteine proteases such as cathepsin B or the Caspase (ICE) family.[7] Given their structural dissimilarity, the ubp and uch families presumably evolved convergently. In general, the uch enzymes are smaller (approximately 30 kDa) and faster, while the ubp enzymes are larger and slower.[29] Also, the ubp family is considerably more diverse. For example, the S. cerevisiae genome contains 16 ubps but only 1 uch.

III. SPECIFIC CELLULAR FUNCTIONS OF DEUBIQUITINATING ENZYMES

As described above, deubiquitinating enzymes act at multiple levels in the ubiquitin pathway (Figure 1). The specific deubiquitinating enzymes acting at each level in the proteasome pathway are largely unknown. For instance, the identity of the enzyme(s) responsible for cleaving the polyubiquitin proprotein (Level 1) is unknown. While Uch enzymes tend to hydrolyze monoubiquitinated substrates (Level 3), little is known regarding their specific substrates or their specific biological activity. Considerably more is known about the enzymes acting at Levels 2, 4, and 5 (see below).

Depending on the level, a deubiquitinating enzyme may promote either the degradation or the stabilization of a given protein substrate. For instance, some deubiquitinating enzymes, such as ubp14, Doa4, and Ap-uch,[19] serve to promote proteasome-mediated degradation of their substrate protein. Other deubiquitinating enzymes, such as the FAF protein and the PA700 isopeptidase,[28] antagonize proteasome-mediated proteolysis. Here, we discuss the characteristics of several specific deubiquitinating enzymes.

A. Deubiquitinating Enzymes That Promote Degradation of Their Substrates

1. Ubp14/Isopeptidase T (Level 2)

One of the best characterized deubiquitinating enzymes is yeast ubp14p and its human homologue, isopeptidase T. Iso-peptidase T is a 93-kDa ubp, first purified from reticulocytes by adsorption to a ubiquitin affinity column.[40] Partially purified iso-peptidase T was originally shown to hydrolyze free polyubiquitin chains[6] and to stimulate degradation of polyubiquitinated protein substrates by the 26S proteasome[17] (Figure 1, Level 2).

Isopeptidase T has well-characterized substrate preferences,[53] as determined *in vitro*. Isopeptidase T hydrolyzes homopolymeric K48-linked ubiquitin chains more efficiently than linear proubiquitin.[53] Furthermore, enzymatic activity is low for substrates containing a ubiquitin chain attached to cytochrome-C or for substrates containing modifications at the C-terminus of the proximal ubiquitin. Taken together, these *in vitro* data strongly suggest that the cellular role of isopeptidase T is to disassemble unanchored polyubiquitin chains at the post-proteasomal level. IsopeptidaseT sequentially degrades these polyubiquitin chains into free monomeric ubiquitin, starting from the free proximal end of the branch chain (Figure 4). Interestingly, the five carboxy-terminal amino acid residues of the 76 amino acid ubiquitin tag contribute to substrate recognition by isopeptidaseT.[44] Also, free ubiquitin inhibits isoT activity with kinetics, suggesting the presence of multiple ubiquitin binding site(s).

Consistent with its observed substrate specificity *in vitro, ubp14* null yeast strains accumulate unanchored polyubiquitin chains.[1] Mutation of *ubp14* also prolongs the half-life of several ubiquitin-pathway substrates, perhaps because the excess ubiquitin chains bind to proteasomes and interfere with proteolysis. Interestingly, despite the fact that *Ubp14* is responsible for most of the soluble cellular deubiquitinating activity against free ubiquitin chains, *UBP14* is not essential for growth. *ubb14* mutant phenotypes were suppressed, however, by human isopeptidaseT, suggesting that this deubiquitinating enzyme has a highly conserved role in the pathway.

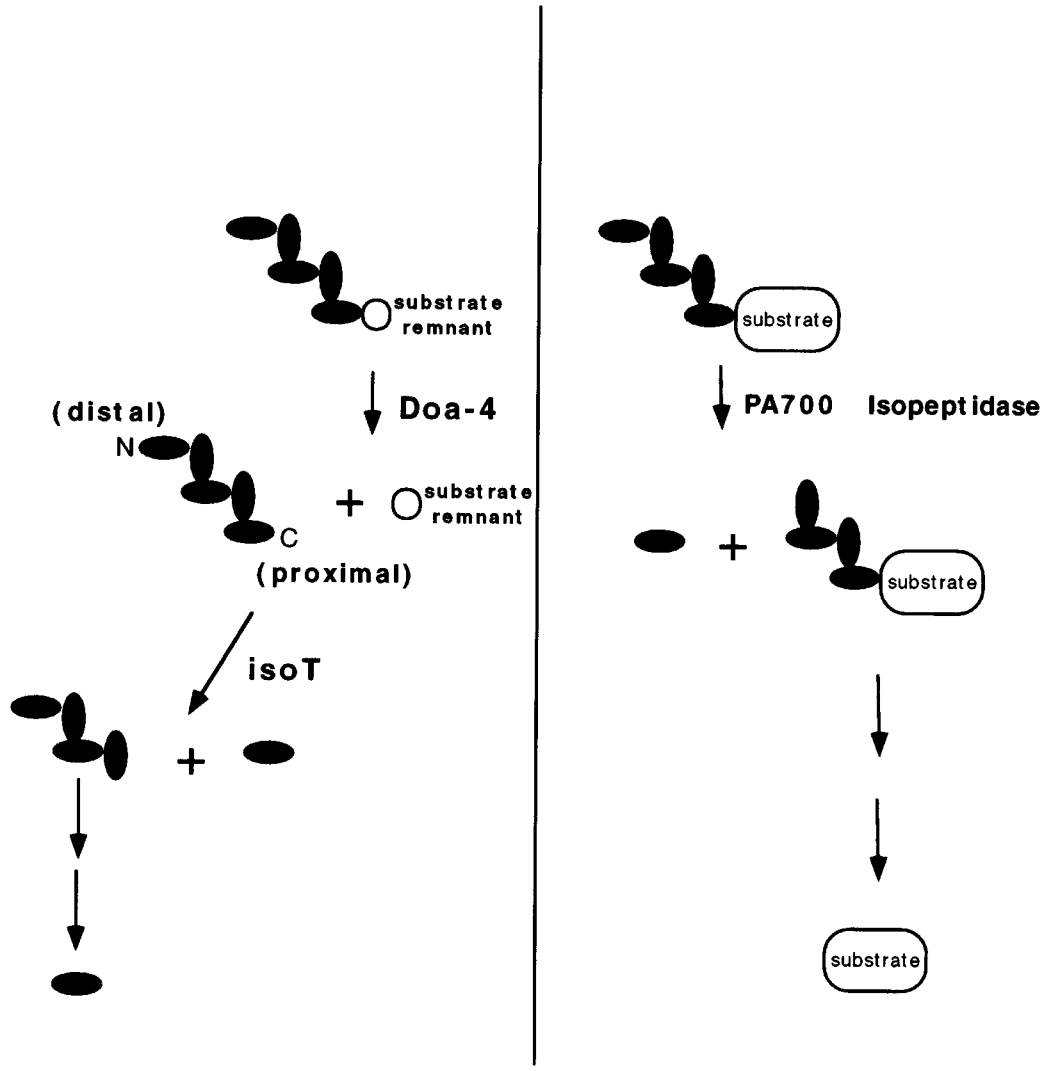

FIGURE 4. Distinct biochemical mechanisms of deubiquitinating enzymes. Shown are the biochemical mechanisms of three deubiquitinating enzymes described in the text. A polyubiquitinated substrate is shown schematically. The carboxy terminal glycine residue (Gly76) of the "proximal" ubiquitin is conjugated through a isopeptide linkage to an internal lysine residue of the protein substrate. Sequential ubiquitin monomers are conjugated via an isopeptide linkage, usually via the Gly76 of one monomer and Lys48 of the next monomer. The "distal" ubiquitin is the last ubiquitin of the branch chain furthest from the substrate. Doa-4 cleaves polyubiquitin conjugates from substrate remnants, presumably by cleaving the isopeptide bond between the proximal ubiquitin and the substrate. Accordingly, small polyubiquitinated substrate remnants accumulate in Doa-4 null mutant yeast strains.[39] Isopeptidase T (IsoT) sequentially cleaves monomeric ubiquitin from the proximal end of a polyubiquitin chain.[53] Branched polyubiquitin chains accumulate in the ubp14/isopeptidaseT null mutant. In contrast, PA700 sequentially cleaves monomeric ubiquitin from the distal end of a polyubiquitin chain bound to a protein target, irrespective of chain length.[28] In this way, PA700 isopeptidase antagonizes proteasome function.

2. Ubp4/Doa4 (Level 5)

Another isopeptidase of the ubp family, the yeast protein Ubp4/Doa4, also serves to promote ubiquitin-mediated proteolysis of some cellular substrates. The primary function of Ubp4/Doa4 appears to be the hydrolysis of isopeptide-linked ubiquitin chains from peptides that are the byproducts of proteasome degradation (Figure 1, Level 5). Genetic analysis has established that this biochemical activity is important for proteasome function *in vivo*. *DOA4*[39] was identified in a screen for mutations that block the degradation of the yeast transcription factor, Matα2.[22] Doa4 has deubiquitinating activity, as assayed both *in vitro* and by cleavage of ubiquitin from a Ub-B galactosidase fusion substrate in *E. coli*. Doa4 mutants exhibit a growth defect, supersensitivity to DNA damage, and impaired degradation of several ubiquitinated substrates. Anti-ubiquitin immunoblotting identifies small peptides bound to ubiquitin polymers that specifically accumulate in the *doa4* mutant. The function of Doa4 therefore may be the clipping of polymeric ubiquitin from peptide degradation products, either at the proteasomal or post-proteasomal level (Figure 4). Whether Doa4 is actually a component of the proteasome has not yet been determined.

While Doa4 clearly promotes the degradation of some cellular substrates, such as Matα2, its precise mechanism remains unclear. Several models are possible. First, Doa4 may deubiquitinate and clear ubiquitinated peptide byproducts resulting from proteasomal degradation of Matα2 (Figure 4). In this way, it would be expected to promote the degradation of only selective substrates. Alternatively, Doa4 may indirectly increase the degradation rate of substrates by performing a more general function, such as increasing the cellular pool of free ubiquitin. Consistent with this latter model, the phenotype of the *doa4* mutant, including its defects in stress response, DNA replication, and receptor internalization, may be accounted for by the resulting depletion of the cellular pool of free monomeric ubiquitin.

3. Ap-uch

Recently, a uch has been identified in Aplysia neurons that, like Doa4 and isopeptidase T, activates proteasomal degradation.[19] This enzyme, termed Ap-uch, is required for a specific long-term memory response. Ap-uch promotes long-term memory by promoting the degradation of a regulatory subunit of Protein Kinase A (PKA) through the ubiquitin-proteasome pathway.[18] Because it promotes degradation, AP-uch also functions at a proteasomal or post-proteasomal level, thereby enhancing the proteolytic activity of its substrate. Interestingly, Ap-uch is induced in response to the stimulus for long-term memory and increases the degradation of specific substrates. *In vitro*, Ap-uch displays the hallmarks of a uch, in terms of its activity against synthetic substrates. Ap-uch is also physically associated with the proteasome and serves to recycle free ubiquitin. Therefore, these experiments connect the regulated expression of a deubiquitinating enzyme and regulated proteasomal activity with an interesting form of physiological control. They also heighten the importance of understanding the biological functions of other inducible deubiquitinating enzymes[57,58] and the human neuron-specific uch, PGP 9.5.[51,52]

B. Deubiquitinating Enzymes That Promote Stabilization of Their Substrates

1. The FAF Protein (Level 4)

Genetic evidence for a deubiquitinating enzyme acting at the preproteasomal level comes from studies of the *Drosophila* ubp encoded by the *fat facets* (*faf*) gene.[12] Flies bearing faf gene mutations have abnormal eyes, containing ectopic photoreceptors in the eye units or facets. Mosaic analysis demonstrated that the FAF protein functions in cells near to but outside the photoreceptors. Therefore, FAF appears to be essential for short-range cell interactions required for fate determination. Interestingly, the FAF protein is 2691 amino acids in length and is the largest known member of the ubp superfamily (Figure 3). The tissue-specific expression of the FAF protein suggests that it plays a highly specific developmental role. Recent evidence demonstrates that FAF function absolutely requires functional deubiquitinating activity.[23] The phenotype of faf mutants is suppressed by a mutation in a proteasome subunit gene. This result argues that the FAF protein deubiquitinates and rescues a ubiquitin-conjugated target, thereby preventing its degradation by the proteasome (Figure 1, Level 4). FAF could either be itself associated with the proteasome, or it could antagonize degradation before the substrate interacts with the proteasome. Also, FAF could rescue the substrate, either by removing the entire isopeptide linked polyubiquitin chain from the substrate, en masse, or by trimming the distal ubiquitination subunits sequentially.

2. PA700 Isopeptidase

Another deubiquitinating enzyme that opposes proteasome degradation is an isopeptidase isolated from the 19S regulatory complex (PA700) of the 26S proteasome.[28] Because the PA700 isopeptidase gene has not yet been cloned, it is not known whether the enzyme is a member of the ubp, uch, or some other deubiquitinating enzyme family. This enzyme, which has a molecular weight of 37 kDa, can hydrolyze multiple substrates, including K48-linked diubiquitin and ubiquitin-conjugated lysozyme or α-globin. Interestingly, the PA700 isopeptidase appears to remove one ubiquitin at a time, starting from the distal end of a polyubiquitin chain, irrespective of chain length (Figure 4). The enzyme is a ubiquitin-specific protease, based on its inhibition by Ub-aldehyde. Ub-aldehyde is a specific active site inhibitor for deubiquitinating enzymes, binding irreversibly to the active cysteine residue of the catalytic domain.[32]

By removing monomeric ubiquitin from polyubiquitinated substrates, the PA700 isopeptidase may provide an editing function for the proteasome. Once ubiquitinated chains have been trimmed, the substrate bind less efficiently to ubiquitin receptors on the 26S proteasome,[9,11] and therefore will be rescued from proteolysis. *In vivo*, PA700 may rescue poorly ubiquitinated or slowly degraded ubiquitinated substrates prior to proteolysis. Therefore, in contrast to isopeptidase T, Doa4, and AP-uch, the PA700 isopeptidase antagonizes proteasome-mediated degradation.

IV. BIOLOGICAL FUNCTION OF OTHER DEUBIQUITINATING ENYMES

There are several deubiquitinating enzymes whose function has been tied to specific biological processes but whose biochemical function in the pathway are not known. These are discussed separately because it is not known whether these

enzymes promote degradation, stabilization, or regulation of their ubiquitinated substrate(s).

A. Deubiquitinating Enzymes and Growth Control

One deubiquitinating enzyme displaying biological activity, namely, transforming activity, is the mammalian oncoprotein, *tre-2*. *Tre-2* is a member of the ubp superfamily[36,37,39] and was originally isolated by transfection of genomic DNA from Ewings Sarcoma cells into 3T3 fibroblasts. Interestingly, the transforming isoform of the *tre-2* oncoprotein is a truncated ubp, lacking the His domain and lacking deubiquitinating activity. In contrast, the full-length *tre-2* protein has deubiquitinating activity but no transforming activity. These results suggest that the wild-type *tre-2* protein acts normally as a growth suppressor within the cell. The truncated (oncogenic form) presumably acts as a dominant negative, sequestering substrates of wild-type *tre-2*. The specific substrate(s) of the wild-type *tre-2* enzyme remain unknown. Recently, another mammalian ubp, *unp*, has also been found to have transforming activity.[16]

A second example of ubps that regulate cellular growth pathways are the *DUBs*.[57,58] *DUB-1* was originally cloned as an immediate-early gene induced by the cytokine IL-3. Several lines of evidence suggest that the *DUB-1* polypeptide plays a growth-regulatory role in the cell. First, the expression of *DUB-1* has the characteristics of an immediate-early gene. Following IL-3 stimulation, the *DUB-1* mRNA is rapidly induced and is super-induced in the presence of cyclohexamide. Second, high-level expression of *DUB-1*, from a steroid-inducible promoter, results in cell cycle arrest prior to S phase. This result suggests that *DUB-1* could control the level of expression or the ubiquitination state of important regulators at the G1/S transition. Finally, the induction of *DUB* proteins may be a general feature of the response to cytokines. Another family member, *DUB*-2, is induced by IL-2.[57] At least four *DUB* genes have been identified to date.[57]

Given their growth-regulatory activity, DUBs may act at many possible levels in the cell. First, *DUB* enzymes may deubiquitinate cell surface growth factor receptors, thereby prolonging receptor half-life and amplifying growth signals.[4,33,35,45,46] Second, *DUB* enzymes may deubiquitinate proteins involved in signal transduction.[25,49] Third, *DUB* enzymes may deubiquitinate cell cycle regulators such as cyclins or cyclin-CDK inhibitors.[38] As for other deubiquitinating enzymes, the current challenge is to identify specific substrate(s).

B. Deubiquitinating Enzymes and Transcriptional Silencing

There has long been a postulated role for ubiquitination in chromatin structure. This idea has been advanced recently by the finding that a mouse homologue of the ubiquitin conjugating enzyme, Rad6, is essential for the maturation of sperm chromatin.[41] Consistent with these observations, recently ubps have been linked to a chromatin regulatory process, transcriptional silencing.[34] Transcriptional silencing in yeast requires the chromatin-associated proteins Sir1, Sir2, Sir3, and Sir4. Recently, Ubp3 was purified in a complex with Sir2, Sir3, and Sir4. Disruption of the *UBP3* locus results in increased silencing, suggesting that Ubp3 is normally an inhibitor of silencing. A general role for ubps in silencing can be inferred from the finding that a *Drosophila* ubp (D-ubp-64E) is also an inhibitor of the analogous process of position-effect variegation.[20]

C. Deubiquitinating Enzymes and Regulation of Viral Infection

Another cellular deubiquitinating enzyme, called HAUSP (for herpes-virus associated ubiquitin specific protease), has been implicated recently in the regulation of Herpes virus infections.[10] Previous studies had shown that the Herpes simplex virus type 1 immediate-early protein, Vmw110, is a potent activator of viral gene expression and is required for the initiation of the viral lytic cycle. The protein translocates to the nucleus of infected cells and localizes to discrete nuclear structures, called ND10, containing the PML protein. Vmw110 binds to a 135-kDa host protein, and this binding is required for its transcriptional activation. Interestingly, the 135-kDa protein turned out to be a novel deubiquitinating enzyme, termed HAUSP. In the absence of viral infection, the host HAUSP protein is normally localized throughout the cell nucleus in a micropunctate pattern. In Herpes-infected cells, the HAUSP transiently binds to the ND10 structures, thereby colocalizing with Vmn110.

The mechanism by which Vmn110 and HAUSP regulate viral infection remains unclear, although several models are possible. For instance, Vmn110 may bind to HAUSP and redirect its enzymatic activity toward the deubiquitination and stabilization of viral proteins required for viral replication. Alternatively, the Vmn110 protein may redirect the HAUSP activity toward the degradation of various host proteins. Identifying the specific substrate(s) of HAUSP and determining the level of HAUSP activity, namely, preproteasomal or postproteasomal, will help resolve these models.

D. Deubiquitinating Enzymes and the Processing of Ubiquitin-like Modifications

Several ubiquitin-like molecules, including UCRP,13 NEDD8, and sentrin/SUMO-1/GMP/Smt3, have been identified recently. All of these ubiquitin-like molecules contain a conserved carboxy terminus, and their attachment to target proteins is through an isopeptide linkage, identical to that of ubiquitin itself. Interestingly, modification with sentrin affects the localization of the target protein. For instance, sentrin-modified target proteins are localized to the nucleus, while UCRP-modified proteins are targeted to intermediate filaments. Each one of these ubiquitin-like proteins presumably has its own subfamily of "ubiquitin" conjugating enzymes (E1, E2, and E3) and perhaps its own subfamily of "deubiquitinating enzyme". For instance, one E2-related enzyme, Ubc9, has a preference for the sentrin tag. An activity that removes sentrin from one substrate (RAN-GAP) has already been identified in crude extracts. The proteins that "deubiquitinate" these new ubiquitin-related molecules have yet to be discovered.

V. SUMMARY AND FUTURE DIRECTIONS

The large number of deubiquitinating enzymes identified to date, including both ubps and uchs, suggests that these enzymes have highly specific roles in regulating various biochemical and biological processes. Deubiquitinating enzymes appear to act in distinct levels in the ubiquitin pathway (Figure 1). Some of these enzymes are thought to work at a preproteasomal level, rescuing ubiquitinated enzymes from degradation, whereas others many act at a proteasomal or

post-proteasomal level, promoting degradation of their substrates. What is known about the enzymatic activity of deubiquitinating enzymes is consistent with their proposed roles at different levels in the pathway. Where it has been tested, deubiquitinating enzymes have distinct substrate preferences. However, although different biochemical activities have been identified there must also be functional redundancy *in vivo* because of the lack of a discernible phenotype in many ubp knockout strains. For any newly discovered deubiquitinating enzyme, the identification of the level of biochemical activity and the relevant substrates are the important priorities.

Various biochemical approaches may be helpful to order to identify the specific substrates of deubiquitinating enzymes. First, substrates may be captured by a biochemical approach. Substrates of specific phosphatases, for example, have been isolated using enzymatically impaired variants.[13] Inactivation of the active cysteine and aspartic acid residues of the catalytic domain of phosphatases results in an inactive or weakly active enzyme capable of stable interaction with substrate. A similar approach may be useful in the isolation of ubiquitinated substrates of deubiquitinating enzymes. Second, clues to the identity of specific substrates may come from the cellular localization of the enzyme or the specific biological effect of overexpressing the enzyme in a cell. A putative substrate of a deubiquitinating enzyme may accumulate in a cell if the deubiquitinating enzyme is knocked out genetically or if a dominant negative form of the enzyme is overexpressed. Third, in a genetically tractable system, such as *Drosophila*, the identification of a specific substrate may come from a suppressor screen of a deubiquitinating enzyme mutant.

Finally, there are many other biochemical levels and substrate candidates for deubiquitinating enzymes that are only beginning to emerge. For instance, while the predominant isopeptide linkage of ubiquitin *in vivo* is via Lys48, other linkages are possible. Ubiquitin must also be processed from preproubiquitin (Level 1) and from ubiquitin conjugated esters and thiol esters. Finally, as described above, subfamilies of "deubiquitinating enzymes" may be specific for the processing of proteins containing ubiquitin-like modifiers, such as SUMO-1, Nedd8, and UCRP. Accordingly, specific deubiquitinating enzymes may prefer to cleave at the site of these novel ubiquitin and ubiquitin-like covalent bonds. With many varied roles in the ubiquitin pathway and now potential roles for these enzymes in ubiquitin-like modifications, deubiquitinating enzymes promise to keep biochemists and geneticists busy for some time to come.

ACKNOWLEDGMENTS

We thank Dan Finley and Chris Larsen for helpful comments on the manuscript. D. P. is supported by a Damon Runyan Scholar Award. A. D. is supported by a Leukemia Society of America Scholar Award.

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