

Conjugation and deconjugation of ubiquitin regulating the destiny of proteins

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Abbreviations: DUB, deubiquitinating enzyme; Ub, ubiquitin

Overview

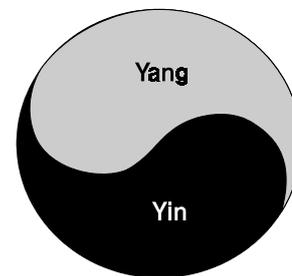
The homeostasis for a number of cellular proteins is regulated by not only phosphorylation and dephosphorylation, but also ubiquitination and deubiquitination. A number of proteins involved in the degradation of polypeptides have been isolated in various eukaryotic organisms from *Saccharomyces cerevisiae* to human. Recently, several deubiquitinating enzymes, classified into either the Ub C-terminal hydrolase (UCH) or the Ub-specific processing protease (UBP), have been reported. It has been shown that they contain conserved domains including Cys, His, and Asp residues throughout the enzyme. These proteins have been demonstrated that Cys and His domains are critical for deubiquitinating enzymatic activity. Recently, we have shown that the Asp domain localized between Cys and His domains is also essential for cleaving the ubiquitin from protein substrates. Mouse deubiquitinating enzymes including DUB-1, DUB-2, and DUB-2A have been isolated and they showed the expression specificity. Of these, DUB-1 and DUB-2 are expressed in lymphocytes depending on the presence of cytokines (interleukin-3 in B-lymphocytes and interleukin-2 in T-lymphocytes, respectively), indicating that they are involved in cytokine signaling pathways. Isolation of all putative DUBs will help to identify their substrates and to regulate the homeostasis of cellular proteins, especially in proliferative cells.

Keywords: lymphocytes; protein processing, post-translational; ubiquitin

Ubiquitin-mediated protein degradation pathway

The concepts of Yin and Yang have provided the intellectual framework of people in Asian countries including Korea, China, and Japan, especially in the fields of biology and medicine. By philosophical analogy, a number of cellular proteins are regulated by not only phosphorylation by kinases, but also dephosphorylation by phosphatases. Recently, a similar counterbalancing action for a number of proteins in eukaryotic organisms is established, ubiquitination by ubiquitin conjugating enzymes and deubiquitination by deubiquitinating enzymes (Figure 1). This includes most of oncoproteins and signaling components involved in receptor tyrosine kinase-mediated signal transduction pathways. Ubiquitin (Ub), a small polypeptide of 76 amino acids, is conjugated to its specific target protein by hierarchical enzymatic reactions (Figure 2). The ubiquitin-activating enzymes (E1) in this cascade reaction activate the ubiquitin molecule in an ATP-dependent manner. This ubiquitin is then transferred to ubiquitin-conjugating enzymes (E2). These enzymes function with ubiquitin-protein ligases (E3) to mediate the final transfer of the ubiquitin to the lysine residues in its protein targets (Hochstrasser, 1996; Wilkinson, 1997; Ciechanover *et al.*, 2000; Weissman, 2001). In yeast, there has been a report that a novel ubiquitination factor (E4) is essential for efficient multiubiquitination (Koepl *et al.*, 1999). Ubi-

Phosphorylation (kinases)
Ubiquitination (ubiquitin conjugating enzymes)



Dephosphorylation (phosphatases)
Deubiquitination (deubiquitinating enzymes)

Figure 1. Like the Yin and Yang mythology in Asian countries, cellular proteins are regulated by not only phosphorylation by kinases and dephosphorylation by phosphatases, but also ubiquitination by ubiquitin conjugating enzymes and deubiquitination by deubiquitinating enzymes.

ubiquitinated proteins are recognized and degraded by the 26S proteasome, which is composed of a proteolytic core catalytic complex (20S) and two 19S regulatory complexes leading to an enormous size (2.5 MDa). These complexes associate together in an ATP-dependent manner (Etlinger and Goldberg, 1977; Herskho, 1996; Holzl *et al.*, 2000; Leggett *et al.*, 2002).

Several classes of proteasome inhibitors have been identified including peptide aldehydes (MG132, PSI, and CEP1612), peptide vinyl sulfone (NLVS), peptide epoxyketones (Epoxomicin and Dihydroeponemycin), peptide boronates (PS-341 and DFLB), and lactacystin and derivatives (lactacystin, omuralide, and PS-519) (Rock *et al.*, 1994; Dick *et al.*, 1996; Tsubuki *et al.*, 1996; Adams *et al.*, 1998; Corey and Li, 1999; Kisselev and Goldberg, 2001). These inhibitors have been used for identifying a number of substrates of the ubiquitin-proteasome pathway and are important for investigating the pathway due to the significance of proteasome in many aspects of cellular protein targets in eukaryotic organisms.

Ubiquitin cleavage by deubiquitinating enzymes

The conjugation of ubiquitin molecule to its specific target proteins is a reversible process. Deubiquitinating enzymes play a role in cleaving the ubiquitin from protein substrates (Figure 2). This cleavage is involved in a number of biologically important processes including preimplantation, growth and differentiation, oncogenesis, cell cycle progression, transcriptional ac-

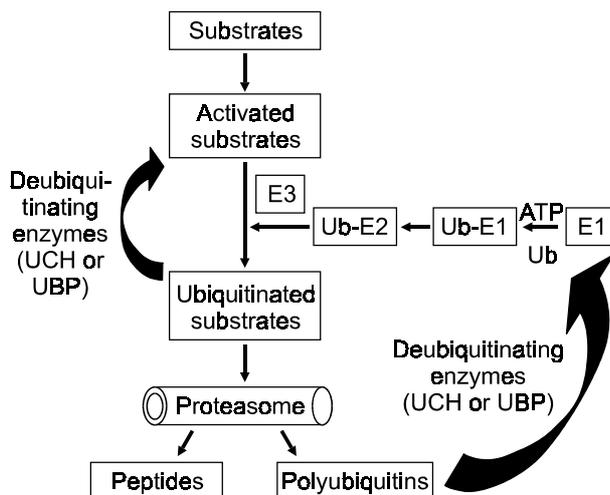


Figure 2. The ubiquitin-proteasome system. Ubiquitin-mediated protein degradation is regulated by a series of Ub-conjugating enzymes (ubiquitin activating enzyme E1, ubiquitin conjugating enzyme E2, and ubiquitin ligase E3) and deubiquitinating enzymes (UCH or UBP). Ubiquitinated protein substrates are degraded by the 26S proteasome.

tivation, and signal transduction (Papa and Hochstrasser, 1993; Moazed and Johnson, 1996; Zhu *et al.*, 1996; Cadavid *et al.*, 2000; Pantaleon *et al.*, 2001; Park *et al.*, 2002). A number of genes encoding a deubiquitinating enzyme have been identified in various eukaryotic organisms and are classified into two groups, the Ub-specific processing protease (UBP) family and the Ub C-terminal hydrolase (UCH) family (Wilkinson, 1997). In general, UBP family members, regulating signal transduction, growth, and development, are responsible for cleaving the ubiquitin from larger proteins and disassembling the polyubiquitin chains. UCH enzymes, playing important roles in development and neural function, are papain-like proteases and are responsible for removing the ubiquitin from small peptides or larger substrates with a flexible peptide linking the C-terminal domain (Wilkinson, 2000).

Seventeen putative deubiquitinating enzymes have been isolated in the genome of *Saccharomyces cerevisiae* (Amerik *et al.*, 2000). This leads to the possibility that a number of deubiquitinating enzymes may exist in higher eukaryotic organisms including human. Recent studies revealed that several deubiquitinating enzymes are involved in cell proliferation and development. This includes the murine *unp* gene that is tumorigenic in nude mice (Gray *et al.*, 1995), the *Drosophila* *faf* gene that determines cell growth and differentiation during eye development in fruit flies (Huang *et al.*, 1995), and the mammalian *tre-2* oncoprotein that shows transforming activity in NIH3T3 fibroblast (Papa and Hochstrasser, 1993). However, the precise molecular mechanisms for these enzymes are not well-studied due to the technical difficulty in purifying ubiquitinated substrates for them. In order to develop pharmacological agents to activate or inhibit proteasomal degradation, isolation and characterization of substrates for deubiquitinating enzymes are required. The human and mouse genome sequencing projects will help to identify genes encoding a putative deubiquitinating enzyme and their substrates in both organisms and are expected to be the largest family members in the ubiquitin system.

Disorders caused by abnormal ubiquitin-mediated protein degradation pathway

It has been demonstrated that aberrant ubiquitin-proteasome pathway can lead to a variety of disorders including neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease (Layfield *et al.*, 2001). In general, protein aggregation is a common symptom for most of the chronic neurodegenerative diseases in humans. The intraneuronal inclusions in many of these diseases include excessive ubiquitin-conjugated proteins, suggesting aberrant ubiquitin-mediated protein degradation.

Parkinson's disease is one of common neurodegenerative disorders characterized by the loss of dopaminergic neurons in the midbrain and the presence of Lewy bodies, cytoplasmic inclusions that are abundantly enriched in ubiquitin (Chung *et al.*, 2001). It has been reported that mutations in the *parkin* gene, encoding an E3 ubiquitin-protein ligase, can cause autosomal-recessive form of juvenile Parkinson's disease (Kitada *et al.*, 1998). Recent studies revealed that parkin protein interacts and ubiquitinates the α -synuclein-interacting protein, synphilin-1 (Chung *et al.*, 2001). Therefore, the aberrant function of parkin, α -synuclein, or synphilin-1 can lead to protein aggregation in Lewy bodies. Another example of protein aggregation is shown in Alzheimer's disease. Mutations in presenilins may lead to early onset forms of Alzheimer's disease due to the disruption of the maturation of the β -amyloid precursor protein (Checler *et al.*, 2000).

Fanconi anemia (FA) is an autosomal recessive cancer susceptibility disease characterized by multiple congenital anomalies, bone marrow failure, and mitomycin sensitivity (Taniguchi *et al.*, 2002; Gregory *et al.*, 2003). A novel FA pathway, required for the normal cellular response to DNA damage, has been suggested (Wilson *et al.*, 2001). Several FA genes including *FANCA*, *FANCC*, *FANCD2*, *FANCE*, *FANCF*, and *FANCG* have been isolated and their gene products interact in a cellular FA pathway (Gregory *et al.*, 2003). It has been demonstrated that *FANCA*, *FANCC*, *FANCE*, *FANCF*, and *FANCG* proteins assemble in a multisubunit complex (Medhurst *et al.*, 2001), and this complex is required for the mono-ubiquitination of the *FANCD2* protein (Wilson *et al.*, 2001). This suggests that the complex plays a role as an ubiquitin ligase. Therefore, the aberrant function of any FA protein in a multisubunit complex may lead to severe clinical damages such as anemia and bone marrow failure.

Deubiquitinating enzymes involved in cytokine signaling pathways

In response to cytokine stimulation, cells change their cellular states. Cytokines induce the expression of specific genes that encode regulatory proteins regulating protein degradation (Akiyama *et al.*, 1994). Recently, the subfamily members of deubiquitinating enzymes (*DUB-1*, *DUB-2*, and *DUB-2A*) have been isolated and have been shown that they are induced by different cytokines in murine hematopoietic cells (Zhu *et al.*, 1996; Zhu *et al.*, 1997; Baek *et al.*, 2001). *DUB-1*, induced by interleukin-3, interleukin-5, or granulocyte-macrophage colony stimulating factor, is an immediate-early gene, and is expressed in murine hematopoietic progenitor cell line Ba/F3 (Figure 3; Zhu *et al.*, 1996). *DUB-2*, is also an immediate-early

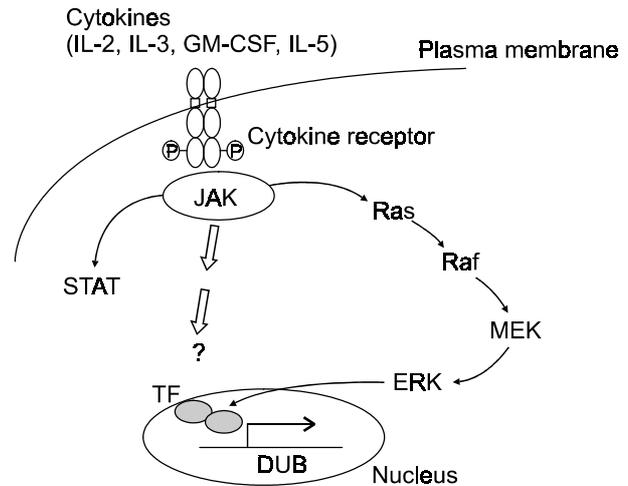


Figure 3. Cytokine-induced JAK activation. JAK kinase is activated upon binding of cytokines to the receptors. They then phosphorylate the receptors on tyrosines, which in turn allow the activation of STAT and Ras signaling. JAK-dependent, STAT-independent pathway is required for the induction of genes encoding murine hematopoietic deubiquitinating enzymes (*DUB-1* and *DUB-2*).

gene, which is induced by interleukin-2 in murine hematopoietic progenitor cell line CTLL (Figure 3; Zhu *et al.*, 1997).

It has been demonstrated that JAK2 kinase and Ras-Raf-MEK-ERK kinase signaling pathway are essential for induction of the murine *DUB-1* gene, even though the presence of another signaling pathway may be involved (Figure 3; Zhu *et al.*, 1997). Interestingly, the minimal interleukin-3 response element of *DUB-1* gene contains cytokine-inducible enhancer activity, but lacks a consensus sequence for STAT binding. This indicates that *DUB-1* is expressed in a JAK2-dependent pathway but STAT5-independent pathway (Zhu *et al.*, 1997). Since a number of receptor tyrosine kinases are regulated not only by phosphorylation and dephosphorylation, but also by ubiquitination and deubiquitination, it is plausible that signaling pathways mediated by these receptors can be modulated by the ubiquitination status of them. In addition, the amount of signaling components, which are to be conjugated with the ubiquitin for proteasomal degradation, can also modulate signaling pathways. Therefore, finding the molecular mechanisms for the regulation of protein degradation via ubiquitination and deubiquitination in RTK-mediated signal transduction pathways will contribute to understanding the regulation of cell proliferation and differentiation.

Structural conservation of deubiquitinating enzymes

Like ubiquitin conjugating enzymes, which are con-

*					
215	GLKNQGATCYMNSLLQTLFF	234	HAUSP	(human)	Cys domain
81	GLQNMGNTCYVNASLQCLTY	100	USP17	(human)	
52	GLQNTGNNSCYLNAALQCLTH	71	DUB-1	(mouse)	
52	GLQNTGNNSCYLNAALQCLTH	71	DUB-2	(mouse)	
52	GLQNTGNNSCYLNAALQCLTH	71	DUB-2A	(mouse)	
* *					
451	HAVLVHSGDNHG-GHYV	466	HAUSP	(human)	His domain
320	YAVLVHAGWSCHNGHYF	336	USP17	(human)	
293	YAVLVHDGATSHSGHYF	309	DUB-1	(mouse)	
293	YAVLVHEGATCHSGHYF	309	DUB-2	(mouse)	
293	YAVLVHEGATCHSGHYF	309	DUB-2A	(mouse)	
*					
475	GKWCKFDDVVSRCCKEE	492	HAUSP	(human)	Asp domain
344	GQWYKMDAEVTSASSITS	361	USP17	(human)	
317	GAWYKMDDTKVTSCDVTS	334	DUB-1	(mouse)	
317	GAWYKMDDTKVTSCDVTS	334	DUB-2	(mouse)	
317	GAWYKMDDTKVTSCDVTS	334	DUB-2A	(mouse)	

Figure 4. Alignment of conserved amino acid sequences for deubiquitinating enzymes derived from GenBank accession numbers, HAUSP (Z72499), USP17 (D38378), DUB-1 (Q61068), DUB-2 (AAB95194), and DUB-2A (AAK84135) using MegAlign software (Clustal method) from DNASTAR (LASERGENE). Conserved amino acid residues for putative catalytic triad according to the structural study are indicated with asterisks.

served from yeast to human, the UCHs of deubiquitinating enzymes are also highly conserved (Johnston *et al.*, 1999). The UBPs contain divergent sequences throughout the enzymes, even though the alignment of them indicates that the six DUB homology domains are found (D'Andrea and Pellman, 1998; Lockington and Kelly, 2001). Of these, three conserved domains (cysteine, histidine, and aspartic acid residues) comprise the catalytic triad for an active site of the enzyme (Figure 4; Baek *et al.*, 2001; Hu *et al.*, 2002). It has been suggested that the divergence of amino acid sequences for UBPs is involved in a broad range of substrate specificity (Papa and Hochstrasser, 1993; Wilkinson, 1997). Site-directed mutagenesis experiments for these conserved amino acids revealed that they are required for deubiquitinating enzyme activity (Figure 5B; Papa and Hochstrasser, 1993; Huang *et al.*, 1995; Baek *et al.*, 2001; Lee *et al.*, 2001; Hu *et al.*, 2002). The deletion analysis of the carboxy-terminal region for deubiquitinating enzyme subfamily members (DUB-1, DUB-2 and DUB-2A) showed no effect for the deubiquitinating enzyme activity (Baek *et al.* 2001; unpublished data). This suggests that the carboxy-terminal region of these enzymes may be involved in substrate specificity, protein-protein interaction, and localization. Recently, the crystal structure for the catalytic domain of HAUSP, one of UBPs that deubiquitinates and stabilizes the tumor suppressor p53, has been studied (Hu *et al.*, 2002). This study showed that the catalytic core domain of HAUSP enzyme binds ubiquitin aldehyde, leading to a dramatic conformational change

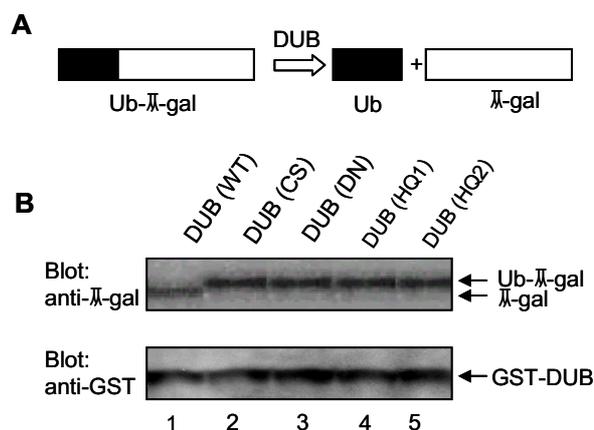


Figure 5. A. Ub- β -gal assay for deubiquitination of ubiquitin- β -galactosidase (Ub-Met- β -gal) fusion protein expressed in *Escherichia coli*. B. The upper panel is an immunoblot using anti- β -gal antiserum. The co-expressed plasmids are pGEX-DUB wild-type (lane 1), pGEX-DUB CS (lane 2), pGEX-DUB DN (lane 3), pGEX-DUB HQ1 (lane 4), and pGEX-DUB HQ2 (lane 5). The lower panel is an immunoblot using an anti-GST monoclonal antibody.

in the active site similar to one for HAUSP binding to its substrate (Hu *et al.*, 2002). The detailed molecular mechanisms for their binding should be investigated in order to create pharmaceutical reagents that target to these mechanisms in signal transduction pathways involved in cell proliferation and differentiation.

Isolation of putative deubiquitinating enzymes and future directions

More than 90 putative deubiquitinating enzymes have been identified by the human genome sequencing project, making them one of the largest family of enzymes in the ubiquitination and deubiquitination system (Chung and Baek, 1999). As mentioned above, the UBP enzymes contain high homology domains that surround the catalytic Cys, His and Asp residues. Using bioinformatics, we previously identified a deubiquitinating enzyme gene, *UBH1*, in mouse testis (Baek *et al.*, 2002). In order to screen putative deubiquitinating enzymes containing a conserved aspartic acid motif for the formation of the oxyanion hole in mouse (Hu *et al.*, 2002), databases from GenBank were accessed and searched using the BLAST algorithm (Altschul *et al.*, 1997) at the National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/BLAST/>). We previously demonstrated for the first time that the conserved aspartic acid residue in the oxyanion hole of mouse DUB-1, DUB-2 or DUB-2A, which is expressed specifically in either B lymphocytes or T lymphocytes, is required for the enzymatic activity (Baek *et al.*, 2001; Lee *et al.*, 2001). By screening the databases, we found a total number of 11 genes encoding a deubiquitinating enzyme including DUB-1, DUB-2, and DUB-2A (Baek *et al.*, 2002). One of them was cloned and named as *UBH1* (Baek *et al.*, 2002). The sequence analysis for these genes suggests a similar mechanism for all UBP enzymes in the formation of the oxyanion hole and catalysis (Hu *et al.*, 2002). The detailed mechanism should be elucidated in order to speculate pharmacological manipulation for UBP-related diseases. Several other putative genes encoding a deubiquitinating enzyme have been identified by this method and they are under investigation.

A novel ubiquitin-specific protease gene *UBP41* was isolated from the chick skeletal muscle cDNA library and the deubiquitinating enzyme activity was analyzed by Ub- β -gal assay in *Escherichia coli* (Figure 5A; Baek *et al.*, 1997). Recently, 23 deubiquitinating enzymes including the tumor suppressor CYLD1 have been identified by chemistry-based functional proteomics in EL4 cells (Borodovsky *et al.*, 2002). In addition, with information obtained from genome sequencing projects in various living organisms including human and mouse, a number of genes encoding a deubiquitinating enzyme will be identified by various methods. While the physiological functions for most of deubiquitinating enzymes *in vivo* remain unknown, several features of them suggest that they play an important role in cell proliferation and differentiation. Therefore, finding specific substrates and the molecular mechanism of deubiquitination by these enzymes will elucidate their physiological roles. Ob-

viously, this will give some insights on how these enzymes may function for intracellular processes including signal transduction pathways, cell cycle progression, transcriptional activation, antigen presentation, apoptosis (or programmed cell death), and DNA repair within the cell.

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