Chemistry & Biology, Vol. 9, 1149–1159, October, 2002, ©2002 Elsevier Science Ltd. All rights reserved. PII S1074-5521(02)00248-X

Chemistry-Based Functional Proteomics Reveals Novel Members of the Deubiquitinating Enzyme Family

Anna Borodovsky,[1,4] Huib Ovaa,[1,4] Nagamalleswari Kolli,[2] Tudeviin Gan-Erdene,[2] Keith D. Wilkinson,[2] Hidde L. Ploegh,[1,3] and Benedikt M. Kessler[1]

[1]Department of Pathology
Harvard Medical School
200 Longwood Avenue
Boston, Massachusetts 02115

[2]Department of Biochemistry
4017 Rollins Research Building
Emory University Medical School
1510 Clifton Road
Atlanta, Georgia 30322

Summary

The ubiquitin (Ub)-proteasome system includes a large family of deubiquitinating enzymes (DUBs). Many members are assigned to this enzyme class by sequence similarity but without evidence for biological activity. A panel of novel DUB-specific probes was generated by a chemical ligation method. These probes allowed identification of DUBs and associated components by tandem mass spectrometry, as well as rapid demonstration of enzymatic activity for gene products whose functions were inferred from primary structure. We identified 23 active DUBs in EL4 cells, including the tumor suppressor CYLD1. At least two DUBs tightly interact with the proteasome 19S regulatory complex. An OTU domain-containing protein, with no sequence homology to any known DUBs, was isolated. We show that this polypeptide reacts with the C terminus of Ub, thus demonstrating DUB-like enzymatic activity for this novel superfamily of proteases.

Introduction

The sequencing of genomes of many organisms provides a wealth of information, the meaningful interpretation of which is a major challenge. Proteins can be classified into distinct families based on sequence similarity, yet this alone does not always accurately predict function. Alternative methods for rapid assignment of biological activity to newly sequenced proteins are therefore required. Sequence comparison for proteins that do not possess enzymatic activity provides information about shared structural elements, such as Ig folds in the Ig superfamily [1]. The roles of such common sequence motifs are frequently divergent and hold few clues to function. Sequence comparison of catalytically active proteins may be more readily interpretable; nevertheless, assignment of a protein to an enzyme class requires the experimental demonstration of its activity. Novel members of an enzyme family can be identified by designing active site-directed probes, which are based on the chemical reactivity toward known target proteins. Such approaches have been utilized to target the serine hydrolases and cysteine proteases [2, 3]. Here, we apply a similar strategy to an enzyme class in the ubiquitin-proteasome system.

The ubiquitin-proteasome pathway has emerged as an essential player in nonlysosomal protein turnover, regulation of the cell cycle, membrane receptor endocytosis, and antigen presentation [4]. Several enzyme families cooperate to tag proteins with ubiquitin (Ub); ubiquitinated proteins are then delivered to the proteasome for degradation or are destined for other cellular fates [5]. Deubiquitinating enzymes (DUBs), which remove Ub from substrate proteins, also regulate the ubiquitin-proteasome system [6]. USP7 (HAUSP) can remove Ub from the p53 tumor suppressor and rescue it from degradation, allowing p53-mediated cell growth repression [7]. Many key cell cycle regulatory proteins are degraded in a Ub-dependent manner [5], and DUBs therefore are likely participants in the regulation of their activity levels. Indeed, several known and putative DUBs, such as CYLD1, BAP1, and Tre-2, are thought to function as tumor suppressors [8-10]. Regulated deubiquitination is likewise observed for proteins that are not targeted to the proteasome. Ub is removed from histone H2A during mitotic and apoptotic chromatin condensation [11, 12]; similarly, Ub is removed from membrane receptors by the yeast Doa4 during endocytosis and targeting to the vacuole [13]. These observations, as well as the tissue-specific expression of many DUBs, suggest that each DUB may be dedicated to a specific substrate(s) [6]. However, the activity of DUBs in complex samples, such as mammalian cells or extracts prepared from them, has been difficult to examine, since many of these enzymes (over 40 in mammalian genomes) are present in the cell.

DUBs belong to two subfamilies of cysteine proteases with no sequence homology. All DUBs are exquisitely specific for the hydrolysis of a peptide bond at the C terminus of Ub. Most DUBs can bind a Ub monomer, even if the monomer is not the preferred substrate in vivo [6]. We therefore designed active site-directed probes that contain an epitope-tagged Ub (HAUb) with a C-terminal thiol-reactive group that can act as suicide substrates. Having shown that ubiquitin vinyl sulfone (UbVS) modifies 6 out of 17 DUBs in budding yeast [14], we designed six additional thiol-reactive groups to target a wider range of DUBs.

Here we report the synthesis of HAUb-derived probes using an intein-based chemical ligation method [15, 16] and show that their reactivity toward DUBs depends on the type of C-terminal electrophile used. Enzymes modified by the HAUb-derived probes were isolated and identified by tandem mass spectrometry. We show that 23 DUBs are targeted by these probes in EL4 cell lysates, including 10 polypeptides for which no enzymatic activity has been previously demonstrated. Furthermore, subunits of the 19S cap of the proteasome can be recovered in association with active DUBs. A gene product suspected of being a thiol protease, with no sequence

[3]Correspondence: ploegh@hms.harvard.edu
[4]These authors contributed equally to this work.

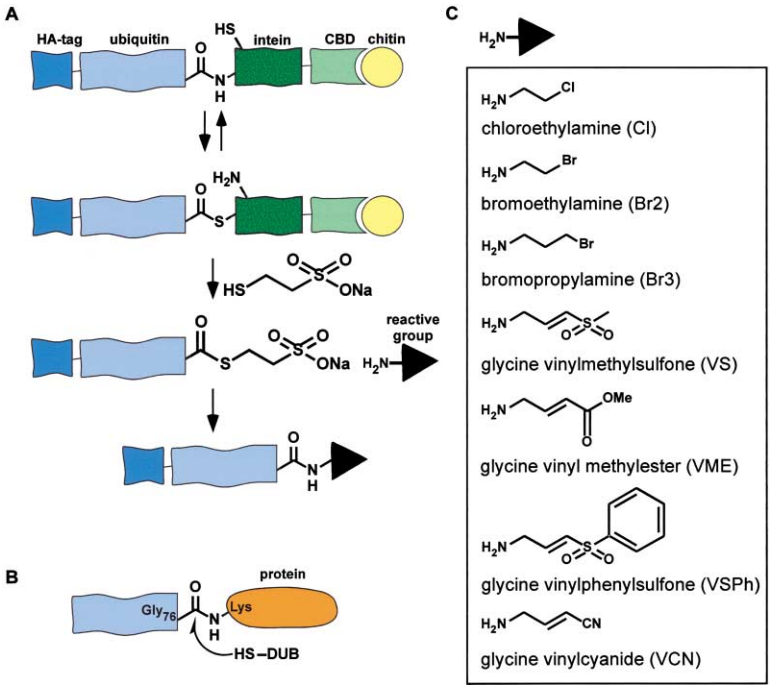

Figure 1. Synthesis of HAUb-Derived Probes

(A) The intein-based chemical ligation method. Recombinant $HAUb_{75}$-intein-chitin binding domain (CBD) fusion protein was bound to a chitin affinity column; on-column cleavage of the HAUb-intein junction was induced by the addition of β-mercaptoethane sulfonic acid (MESNa). The resulting $HAUb_{75}$-MESNa thioester was reacted with a desired C-terminal thiol-reactive group as described in Experimental Procedures, generating the desired HAUb-derived probe.

(B) Site of attack of a hydrolase on the peptide bond at the C terminus of Ub.

(C) Structures of C-terminal thiol-reactive groups used.

homology to DUBs (containing an ovarian tumor [OTU] domain [17]), was also found to be reactive with C-terminally modified Ub. This approach can be extended to any class of enzymes that can be targeted covalently and help assign enzymatic activity to new gene products.

Results

Synthesis and Characterization of HAUb–Derived Probes

The strategy for the synthesis of active site-directed probes is outlined in Figure 1A. N-terminally HA-tagged Ub (HAUb) lacking Gly76 was expressed in *E. coli* as a fusion protein with an intein and a chitin binding domain [16]. Purification over chitin-beads, followed by trans-thioesterification led to the isolation of the desired thioester ($HAUb_{75}$-MESNa). Desired irreversible inhibitors were synthesized by chemical ligation of the reactive groups (Figure 1C) with $HAUb_{75}$-MESNa in 50%–90% yield and purified by cation-exhange chromatography. The molecular weights of the pure HAUb derivatives were in agreement with the predicted masses, as assessed by mass spectrometry (LC-ESI-MS, see Supplemental Data). The intein-based chemical ligation approach is amendable to the introduction of diverse chemical groups at the C terminus of Ub and gives products of greater purity and at higher yield then the reverse trypsinolysis method used previously [14].

Seven HAUb-derived probes with different C-terminal electrophilic traps were generated (Figure 1C), including four Michael acceptor-derived probes [18, 19], vinyl methyl sulfone (HAUbVS), vinyl methyl ester (HAUbVME), vinyl phenyl sulfone (HAUbVSPh), and vinyl cyanide (HAUbVCN), and three alkylhalide-containing inhibitors, chloroethyl (HAUbCl), bromoethyl (HAUbBr2), and bromopropyl (HAUbBr3). The use of simple alkylhalides as protease inhibitors has not been previously reported. All probes (except HAUbBr3) were designed to react at a position that corresponds to the C-terminal carbonyl of the Gly76 amide bond conjugating Ub to its substrate (Figure 1B). Based on their chemical reactivity, the electrophiles used are expected to differ in their ability to modify DUBs and consequently may selectively label different molecular targets. Recombinant, purified UCH-L3 enzyme [20] was reacted with the HAUb-derived probes. An additional polypeptide of a molecular mass consistent with covalently modified UCH-L3 was observed for all probes, confirming their ability to target deubiquitinating enzymes (data not shown). Full-length HAUb was also generated by chemical ligation with glycine and was shown to be incorporated into poly-Ub conjugates (Figure 2, lane 2). Thus, the presence of an HA tag does not adversely affect interaction with enzymes that utilize Ub.

Profiling Enzymes Modified by HAUb-Derived Probes in EL-4 Cell Extracts

To address the specificity of the HAUb-derived probes toward DUBs, we carried out labeling experiments using whole-cell lysates. The EL4 mouse thymoma cell line was selected for this purpose, since it expresses a diverse set of deubiquitinating enzymes [14]. The enzymes modified were visualized by anti-HA immunoblotting after incubation with HAUb-derived probes (Figure 2). We observed distinct profiles of labeled polypeptides for different Ub C-terminal thiol-reactive groups used. In all cases, labeling could be blocked by inclusion of the alkylating agent N-ethyl-maleimide (NEM) (data not shown), consistent with the presence of an active site cysteine residue in the enzymes modified. Addition of HAUb to the lysate (Figure 2, lane 2) resulted in efficient

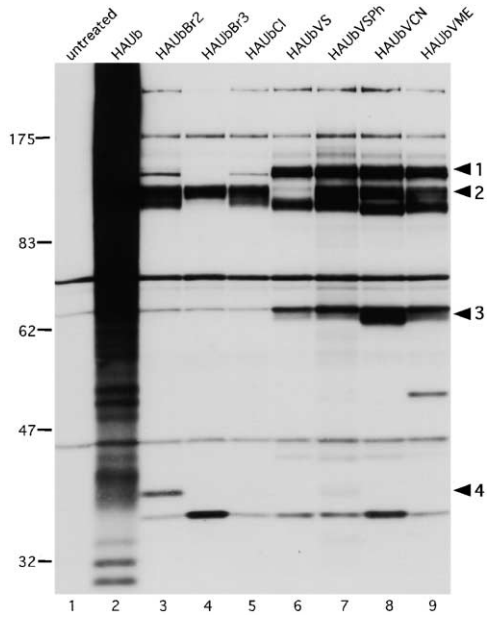

Figure 2. Different HAUb-Derived Active Site-Directed Probes Show Distinct Labeling Profiles

EL4 cell lysates (20 μg) were incubated with 0.5 μM of HAUb HAUbVS, HAUbVME, HAUbVSPh, HAUbBr2 or 1 μM of HAUbCN, HAUbCl, HAUbBr3 as indicated. The labeled proteins were resolved by 8% reducing SDS-PAGE and immunoblotted with anti-HA antibody. Polypeptides referred to in the text are indicated by arrowheads.

formation of poly-Ub conjugates, while such high molecular weight conjugates were not observed upon treatment of the lysate with HAUb-derived probes.

Treatment of the lysate with HAUbVS (Figure 2, lane 6) produced a labeling pattern similar to that observed with [^{125}I]-UbVS [14], further confirming that the HA tag does not hamper the recognition of HAUbVS by DUBs. Inspection of the profile in Figure 2 shows comparable labeling intensity for the cluster of polypeptides around MW 140 kDa (arrow 1) for all probes containing a Michael acceptor (Figure 2, lanes 6–9). For these proteins, the nature of the electrophilic substituent does not affect labeling efficiency. In contrast, for the cluster of polypeptides at MW 130 (Figure 2, arrow 2), significant differences in labeling efficiency are observed when different Michael acceptors are used. Therefore, for a given DUB, not all probes are equally effective (see also Figure 2, arrow 3). The labeling pattern was relatively insensitive to the size of the substituent present in the Michael acceptor compounds (compare HAUbVS and HAUbVSPh).

The alkyl halide-containing probes (HAUbBr2, HAUbCl, HAUbBr3) modify a subset of the polypeptides labeled by the Michael acceptors, with the single exception of a unique polypeptide around 40 kDa modified by HAUbBr2 (Figure 2, arrow 4). In this case, it cannot be excluded that the labeling takes place via an aziridine intermediate. Interestingly, HAUbBr3, in which the reactive position is one carbon removed from the preferred site of attack by a hydrolase, displays more restricted reactivity, modifying only 4 distinct proteins (Figure 2, lane 4). This suggests that precise positioning of the thiol-reactive group in the active site is necessary for optimal reactivity and that this property may be exploited to achieve selectivity.

Enzymes Modified by HAUb-Derived Probes Are Part of Multiprotein Complexes

To isolate the proteins modified by HAUbVS, we immunoprecipitated labeled proteins from EL4 lysates using anti-HA antibody bound to agarose beads as outlined in Figure 3A. EL4 lysates were incubated with HAUbVS and either denatured with SDS to dissociate noncovalent protein complexes or left untreated. These samples were then subjected to immunoprecipitation with an immobilized anti-HA antibody.

Modification with HAUbVS (denaturing conditions, Figure 3B, lane 3) resulted in a labeling profile similar to that observed in anti-HA immuno-blots (Figure 2, lane 6). Recovery of most immunoprecipitated polypeptides was abolished by inclusion of a 3-fold molar excess of untagged UbVS, demonstrating specificity for UbVS. Samples immunoprecipitated under native conditions (Figure 2, lane 4) contained not only the proteins observed in the denatured sample, but also more than 10 additional prominent polypeptides (Figure 3B, lane 4, indicated with asterisks). Competition with untagged UbVS (Figure 3B, lane 5) reduces their recovery, showing that these proteins associate specifically with enzymes modified by HAUbVS.

Identity of Enzymes Modified by HAUbVS and Their Associated Factors

In yeast, the availability of deletion mutants allowed the identification of all targets of [^{125}I]-UbVS [14]. While radiolabeled [^{125}I]-UbVS allowed us to demonstrate the multiplicity of mammalian targets for this probe, the identity of most modified polypeptides could not be established. Incorporation of an HA epitope tag into UbVS allows the retrieval of covalently modified enzymes for identification. The identity of polypeptides from a HAUbVS-treated, nondenatured sample is shown in Figure 3C and Tables 1 and 2. In all, 16 DUBs and 12 of the 18 known subunits of the 19S lid and base were identified (Figure 3B; Tables 1 and 2). The recovery of 19S cap subunits is in agreement with the known binding of USP14 and UCH37 (both labeled by HAUbVS) to the 19S complex [14, 21, 22]. Association of other labeled DUBs with the 19S regulatory complex cannot be excluded at this point.

Some DUBs were identified in several forms differing in their molecular weight (Table 1). Modification by HAUbVS is predicted to increase the size of a DUB by approximately 10 kDa and the resulting branched polypeptide may migrate at a larger apparent molecular weight. The presence of different molecular weight species may be also due to conjugation of Ub to the HAUbVS-modified protein, since polyubiquitination occurs under the conditions used in the assay (Figure 2A, lane 2). Proteolysis is unlikely to be a significant factor, since most observed molecular weights exceed the masses predicted from primary sequence. Proteins not currently linked to the ubiquitin-proteasome system were also recovered under native conditions (Figure 3C;

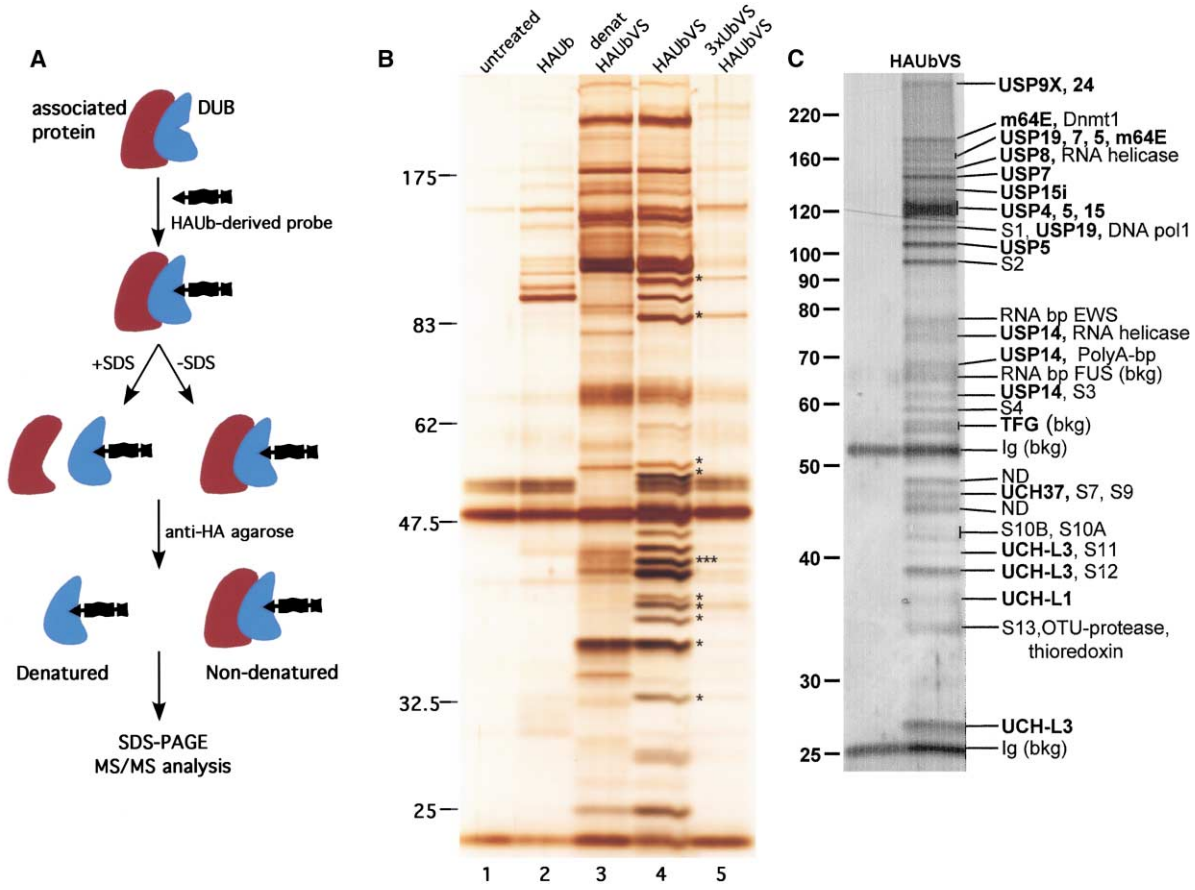

Figure 3. Protein Complexes Containing HAUbVS Modified Enzymes Can Be Recovered by Anti-HA Immunoprecipitation

(A) Outline of the immunoprecipitation experiment to recover proteins covalently modified by HAUbVS with (nondenatured) or without (denatured) their interacting partners.

(B) Silver stain of HAUbVS modified proteins under different conditions. Five milligrams of lysate were treated with 6.6 μg of HAUb (lane 2), HAUbVS (lane 3), and a 3-fold molar excess of untagged UbVS as a competitor (lane 5). "Denatured" samples (lane 4) were treated with 0.4% SDS prior to the addition of anti-HA agarose. Bound proteins were eluted with 50 mM glycine (pH 2.5), resolved by 8% reducing SDS-PAGE, and silver stained. 19S subunits are indicated by asterisks.

(C) Identity of enzymes and associated proteins bound by HAUbVS. Nondenatured samples treated with HAUbVS were prepared as described in Figure 3. Silver or Coomassie stained bands were excised from gels, proteins were in-gel digested with trypsin and the eluted peptides were sequenced on a Micromass MS/MS Q-TOF MICRO instrument. Proteins were identified by searching the results against the SwissProt, Tremble, and TrembleNew databases using the ProteinLynx GlobalServer software 1.1 (Micromass). Any matches with scores of 100 or above were considered significant. Alternatively, the obtained MS results were search against the NCBInr database using Mascot (MatrixScience). Deubiquitinating enzymes are indicated in bold; ND, not determined; bkg, background.

Table 2). A number of these polypeptides interact with RNA. RNA binding hnRNP proteins and ribosomal subunits are common contaminants in affinity-purified material [23] and are recovered in our experiments. Most of the identified proteins are linked to RNA processing and transport, and some of them might indeed interact directly with DUBs.

DUBs Exhibit Selectivity for Different HAUb-Derived Probes

HAUbVS appears to be highly specific for DUBs. However, introduction of other chemical substituents at the C terminus of Ub may allow the modification of additional enzyme classes. To address this question, we identified the enzymes modified by HAUbVME and HAUbBr2, since the labeling patterns for these inhibitors deviate significantly from that observed for HAUbVS (Figure 2). Figure 4 shows the profile of polypeptides immunoprecipitated from lysates treated with HAUbVS, HAUbVME and HAUbBr2 under native (Figure 4A) or denaturing (Figure 4B) conditions. As observed in immunoblot (Figure 2), HAUbVME immunoprecipitates a larger number of proteins than HAUbVS (Figure 4B, compare lanes 3 and 4). HAUbBr2 shows a more restricted labeling profile with a unique polypeptide at 42 kDa (Figure 4B, lane 5, arrowhead). MS/MS analysis showed that HAUbVME modifies USP11, USP12, USP13, USP25, USP28, CYLD1, and a USP fragment, KIAA891, in addition to all the DUBs labeled by HAUbVS (Table 1). Despite the larger number of DUBs labeled by HAUbVME, the majority of the associated proteins recovered in the nondenatured samples appear to correspond to the subunits of the 19S proteasome cap as judged from the silver-stained sample (Figure 4A, lane 4).

Table 1. Enzymes Modified by HAUb-Based Probes

Protein	Accession Number	Predicted MW (kDa)	Observed MW	Number of Matches	Sequence Coverage (%)	Denatured	Nondenatured	Ub Derivative Modified by	Activity Demonstrated	Remarks
USP4 (Unp)	P35123	108	120, 130, 140	14	14	+	+	VS, VME, Br2	[57]	binds pRb, p107, p130 [38]
USP5 (IsoT1)	P56399	95.8	120, 130, 140, 170, 220	18	24.3	+	+	VS, VME, Br2	[39]	disassembles free poly-Ub chains [39]
USP7 (HAUSP)	Q93009 (h)	128	140,150, 170	11	12.2	+	+	VS, VME, Br2	[58]	binds ICP0 (HCMV), TRAF 1-6; binds and deubiquitinates p53 [7, 40, 41]
USP8 (Ubp-Y)	Q9EQU1	122.5	155	10	30.2	+	+	VS, VME	[42]	binds Ras-GRF1, Hbp; levels increase with growth stimulation [42-44]
USP9X (FAFX, FAM)	P70398	290	300	22	11	+	+	VS, VME, Br2	[45]	Regulates ß-catenin and AF-6, fly homolog deubiquitinates liquid facets [45, 46]
USP10	P52479	87	120, 140, 150	4	5.1	+	+	VS, VME	[47]	G3BP binding inhibits activity [47]
USP11	P51784 (h)	79	130	3	5	+	ND	VME	[48]	binds RanBPM [48]
USP12 (UBH1)	Q9D9M2	41	39, 50	1	3.7	+	ND	VME	[59]	
USP13 (IsoT3)	Q92995 (h)	97.3	120	3	2.9	+	ND	VME	**this study**	54% identical to USP5 [49]
USP14 (TGT)	Q9JMA1	56	62, 69, 75,79	6	12.8	+	+	VS, VME	[60]	Proteasome bound; activity modulated by 26S association [14,50]
USP15	Q9Y4E8 (h)	103	120, 130, 140	12	14.4	+	+	VS, VME, Br2	[51]	60% identical to USP4 [51]
USP15i	Q9Y5B5 (h)	112	140, 130	6	6.4	+	+	VS, VME, Br2	**this study**	splice variant of USP15
USP16 (Ubp-M)	Q99LG0	93	140	2	2.7	+	ND	VS, VME, Br2	[52]	de-Ub histone H2A; binds chromatin; phosphorylated during the cell cycle [52]
USP19	O94966 (h)	151	120, 170, 200	5	6.3	+	+	VS, VME, Br2	**this study**	
USP24	Q9UPU5 (h)	112	300?	6	5.4	+	ND	VS, VME, Br2	**this study**	
USP25	P57080	121		9	13.7	+	ND	VME	**this study**	
USP28	Q96RU2 (h)	122.5	150	3	3.2	+	ND	VME	**this study**	
CYLD1	Q96EH0 (h)	107	120	4	4.5	+	ND	VME	**this study**	tumor suppressor gene mutated in cylindromatosis [8]
m64E	Q96K76	147	170, 200	12	9.4	+	+	VS, VME, Br2	**this study**	mutant of fly homolog enhances position effect variegation [53]
USP frag 1 KIAA891	Q96PZ6	87 (frag)	140	2	5.5	+	ND	VME	**this study**	fragment
UCH-L1	Q9R0P9	24.8	37	2	9.4	+	+	VS, VME	[61]	linked to Parkinson's disease, mutated in gracile axonal dystrophy in mice [6, 54]
UCH-L3	Q9JKB1	26	31, 39, 41	11	57	+	+	VS, VME, Br2	[61]	cleaves Ub-gene products, KO mouse has no phenotype [55, 56]
UCH37	Q9WUP7	37.6	39, 48, 55	4	13.1	+	+	VS, VME	[22]	19S cap subunit; edits poly-Ub chains [21, 22]
HSPC263 (OTU-protease)	Q9P0B8	31.6 (frag)	42, 35 (VS, unmodified)	4	16.2	+	ND	Br2	**this study**	An unmodified version is detected in non-denatured HAUbVS immunoprecipitations

Proteins were isolated and sequenced as described in the text. Peptide matches were assigned using the ProteinLynx Global server software (Micromass); any match with a score greater then 100 was considered significant. SwissProt accession numbers are for mouse sequences unless indicated otherwise ("h" indicates a human sequence where a mouse sequence could not be found). Observed molecular weights correspond to proteins modified by HAUb-derived probes (adds 10 kDa); see text. Number of matches gives the number of peptides identified for each protein. For proteins detected multiple times, the highest number of matches is given. Percent sequence coverage is given for the greatest sequence coverage observed. Denatured samples were analyzed for all three probes; nondenatured samples were analyzed for HAUbVS-treated samples only. Enzymes whose activity was demonstrated in this study are indicated in bold. References to studies that have demonstrated enzymatic activity previously are given. ND, not determined.

Table 2. Proteins That May Associate with DUBs

Protein	Accession Number	Predicted MW (kDa)	Observed MW (kDa)	Number of Matches	Sequence Coverage (%)	Remarks
S1 (Rpn2)	Q99460 (h)	106	115	3	6.2	19S cap subunit (base)
S2 (Rpn1)	Q13200 (h)	100	97	18	26.2	19S cap subunit (base)
S3 (Rpn3)	P14685	60.7	61	16	33.2	19S cap subunit (lid)
S4 (Rpt2)	Q03527 (h)	49	59	12	33.6	19S cap subunit (base)
S7 (Rpt1)	P46471	48.5	48	4	10.2	19S cap subunit (base)
S9 (Rpn6)	O00495 (h)	47.4	48	1	2.8	19S cap subunit (lid)
S10B(Rpt4)	Q92524 (h)	44	43, 44	8	26.7	19S cap subunit (base)
S10A (Rpn7)	Q99JI4	45.5	44	20	47.3	19S cap subunit (lid)
S11 (Rpn9)	Q9WVJ2	42.8	41	16	43.4	19S cap subunit (lid)
S12 (Rpn8)	P26516	36.5	39	3	15	19S cap subunit (lid)
S13 (Rpn11)	O35593	34.5	34	1	4.2	19S cap subunit (lid)
DNA methyltransferase	P13864	183	200	5	16.4	Dmnt1
DNA pol1 subunit	CAC96831	98.5	115	1	1.1	
RNA helicase A	O70133	149	150	10	8	binds mRNA[a]
RNAbp EWS	Q01844	68	75	1	2.1	binds mRNA[a]
PolyA-BP	P11940	70	69	13	24.5	binds mRNA[a]
RNA helicase PL10	P16381	73	73	2	3.9	binds mRNA[a]
thioredoxin-like	CAC40691	37	34	1	3.9	
aminotransferase	Q98JR5	44	39	1	2	

[a]InterPro database (http://www.ebi.ac.uk/interpro/).

Proteins were isolated from nondenatured samples modified with HAUbVS and sequenced by MS/MS as described in the text. Only proteins absent from the denatured preparations are given; those occurring in both denatured and nondenatured samples were considered nonspecific. Accession numbers, number of matches, and percent coverage are as in Table 1. 19S lid and base assignments are based on [29].

Interestingly, 19S subunits were not recovered from nondenatured samples treated with HAUbBr2 (Figure 4A, lane 5). This is consistent with the inability of HAUbBr2 to modify the proteasome bound DUBs UCH37 and USP14 (Figure 4A, asterisks). The denatured and nondenatured HAUbBr2 samples (compare lane 5 in Figures 4A and 4B) look remarkably similar, indicating that enzymes labeled by HAUbBr2 do not have strong noncovalent interacting partners. The unique 42 kDa polypeptide present in both denatured and native samples was identified as a predicted protein containing an ovarian tumor (OTU)-like domain (HSPC263, Table 1). This domain is present in a variety of proteins and has been predicted to encode a novel cysteine protease signature [17]. Its modification by HAUbBr2 is the first demonstration of enzymatic activity for an OTU-domain containing protein, such as HSPC263. Labeling of this polypeptide is diminished by NEM treatment and competed by inclusion of 100-fold excess of unmodified Ub (data not shown), consistent with a requirement for an active site cysteine and at least some measure of specificity for Ub.

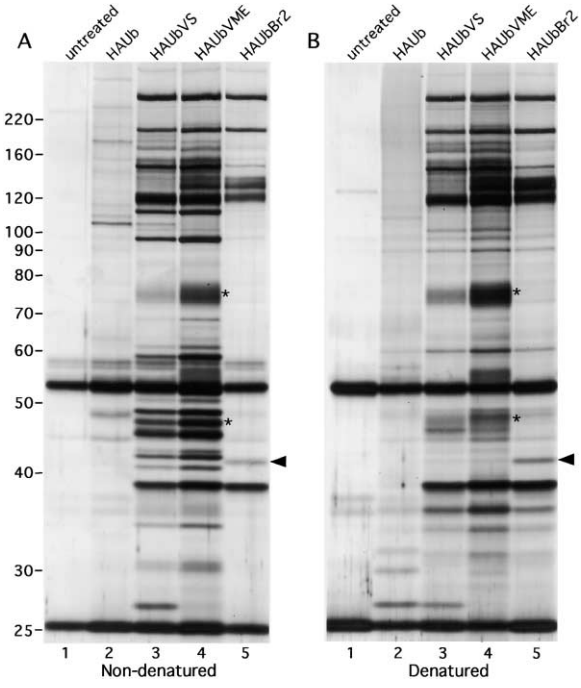

Figure 4. Proteins Bound by HAUbVME and HAUbBr2 under Nondenaturing (A) or Denaturing (B) Conditions

Samples were prepared as described in Figure 3; equal amounts of different inhibitors were used in all reactions. Proteins were visualized by silver stain. Known proteasome-associated DUB are indicated by asterisks; OTU-protease is indicated by an arrowhead.

Discussion

The Ub-proteasome system includes a large number of deubiquitinating enzymes, many of them known by sequence similarity only. The biological role of most of these enzymes remains unknown. Elucidation of the function of individual DUBs is complicated by a considerable overlap in substrate specificity as indicated by deletion studies in yeast, in which a high level of redundancy between DUBs is observed [24] (R. Casagrande, A.B., and H.P., unpublished data). Alternative approaches are clearly required, and a step in this direction is the development of DUB-specific inhibitors based on C-terminal modifications of Ub [14, 21, 25].

We identify DUBs on the basis of their reactivity toward suicide substrates composed of three elements: the Ub moiety which confers specificity for the DUB

family, a thiol-reactive group that allows covalent mechanism-based trapping of the active site cysteine, and an epitope tag that allows nonradioactive detection of modified DUBs as well as their isolation and subsequent identification. The use of several different electrophilic moieties allowed us to target enzymes that do not react with UbVS [14].

We observed that different electrophiles at the C terminus of Ub vary considerably in their ability to react with individual DUBs (Figure 2), indicating that the active sites of DUBs belonging to the same family are not equivalent and that it may ultimately be possible to design more selective probes. As suggested by the polypeptide profile in immunoblots, HAUbVME exhibited the broadest reactivity, while HAUbBr2 modified a more restricted number of DUBs as well as a novel protein not related to DUBs by sequence. The enzymatic activity of nine proteins, predicted based on sequence homology, could thus be established experimentally.

Tandem mass spectrometry-based analysis indicated that HAUb-derived probes modify 3 members of the Ub C-terminal hydrolase (UCH) and 20 members of the Ub-specific protease (UBP) families of DUBs in EL4 cells (Table 1). At least 1 other UCH–BAP1 and as many as 20 other UBPs are encoded by the mouse (and human) genome [6]. We analyzed the sequences of the UBP family enzymes to determine whether the UBPs modified by HAUb-derived probes share features that can account for their reactivity. Figure 5 compares the 34 UBPs annotated by SwissProt and GenBank databases, a subset of which was identified in our experiments as active DUBs. A phylogenetic tree derived from an alignment of the catalytic domains shows that the UBPs modified by all three inhibitors (shaded boxes) form several clusters. Not all enzymes within a particular cluster are labeled by the same inhibitors; for instance, USP5 is modified by HAUbVS, HAUbVME, and HAUbBr2, while a related enzyme, USP13, is targeted by HAUbVME only (Table 1). No significant shared sequences (other than those common to all UBPs) were detected among the 20 UBPs labeled by HAUb-derived probes, suggesting that no additional unique motifs are required for modification of UBPs by HAUb-derived probes. The lack of shared sequence determinants is underscored by the modification of CYLD1 (a tumor suppressor mutated in cylindromatosis [8]) by HAUbVME despite its limited similarity to the UBP family.

Labeling of extracts from other cell lines shows different profiles of targeted polypeptides (data not shown), suggesting that some of the UBPs that we do not detect are simply not expressed in the cell line used in this study. The lack of labeling of some of the UBPs may also be due to low affinity of certain enzymes for monomeric Ub. A subset of DUBs may not interact with Ub at all, but have Ub-like proteins as their true substrates, as has been reported for USP18, which is specific for UCRP [26] and is not modified by our probes.

Most polypeptides modified by HAUb-derived probes contain known sequence motifs characteristic of the UBP or UCH enzymes families, but other enzymes that can react with the C terminus of Ub may exist. One such protein (HSPC263) is modified by HAUbBr2. This protein contains a conserved domain present in 80 other proteins, termed the ovarian tumor domain (OTU) after the *Drosophila* ovarian tumor gene in which it was initially characterized [27]. None of the OTU domain proteins have known biochemical function, but a sequence comparison study identified a novel cysteine protease signature within this domain, which shares limited homology with the papain-type proteases [17]. The HSPC263 protein contains conserved residues predicted to form the active site of this protease family (Asp92, Cys95, and His269), and its modification by HAUbBr2 is inhibited by alkylation with NEM, showing that modification is dependent on an active site cysteine. This is the first demonstration of enzymatic activity for this novel protease superfamily. HSPC263 is also detected in nondenatured samples treated with HAUbVS. In this case, the protein is not modified by HAUbVS, since it is found at its predicted molecular weight of 32 kDa. This observation suggests that HSPC263 is part of a complex with an active DUB. While HSPC263 does not contain any other recognizable domains, several proteins containing an OTU domain possess UIM, UBA, or Ubl domains, and in one case, a catalytic signature of a UBP [17] (available also in the InterPro database: http://www.ebi.ac.uk/interpro/). This suggests a further link of the OTU superfamily to the Ub system.

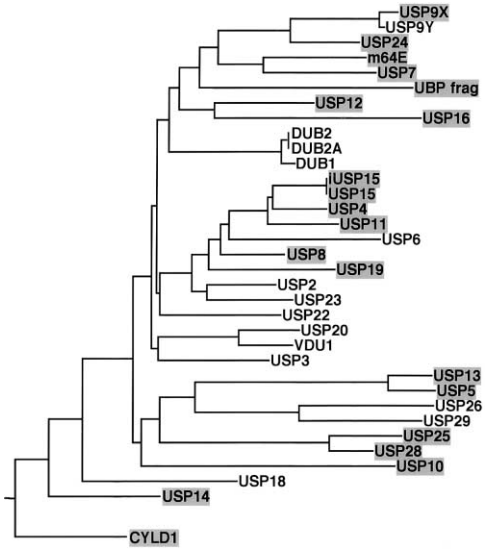

Figure 5. Sequence Comparison of the UBP Family

Catalytic domains of the UBPs annotated in SwissProt or GenBank databases or sequenced in our analysis were assigned based on ProSite parameters (http://us.expasy.org/prosite, profile PS50235). Catalytic domains were aligned using MegAlign program of the DNAStar software package (using a Clustal V algorithm PAM250 matrix); all catalytic residue signatures were well aligned, except CYLD1, for which a recognizable His box could not be found. A neighbor-joining phylogenetic tree [37] was then generated based on the alignment. Enzymes targeted by HAUb-derived probes (HAUbVS, HAUbVME, and HAUbBr2) are shaded. Accession numbers of labeled UBPs are given in Table 1, accession numbers of unmodified UBPs are as follows (SwissProt accession numbers are given, unless indicated otherwise, h-human sequence): USP9Y-h (O00507), DUB2 (O55190), DUB2A (Q923V2), DUB1 (Q61068), USP6 hr (P35125), USP2 (O88623), USP23 (Q9QZL6), USP22 hr (Q9UPT9), USP20 hr (Q9Y2K6), VDU1 (AAL78316-GenBank), USP3 hr (Q9Y6I4), USP26 (Q99MX1), USP29 (NP_067298-GenBank), USP18 (Q9WTV6).

All proteins covalently modified by HAUb-derived probes are hydrolases. Ub-activating and conjugating enzymes are not targeted by our probes. The chemistry of the E1 and E2 enzymes and their low affinity for free Ub makes them poor candidates for modification with HAUb-based probes (for review see [5]). The HECT-domain E3s are the only other enzymes in the Ub system known to form a covalent thioester bond with the C terminus of Ub [28]. Modification of the purified HECT domain of E6-AP by UbVS is in fact observed in the presence of excess inhibitor (data not shown). Nevertheless, we do not recover E6AP or related E3s using HAUb-based probes, suggesting that under our experimental conditions their modification is undetectable in cell lysates, where enzymes with higher affinity for Ub and greater reactivity toward the probes would react preferentially.

Affinity purification of DUBs under native conditions allows recovery of associated proteins (Figure 3). The majority of polypeptides recovered together with DUBs modified by HAUbVS and HAUbVME are subunits of the 19S cap of the proteasome. DUBs are known to associate with the 26S proteasome and are thought to remove poly-Ub chains from proteins targeted for degradation. While several DUBs may bind the proteasome [29], only UCH37 and USP14 have been shown to bind the 26S complex in a stable fashion and may be responsible for editing poly-Ub chains and Ub recycling, respectively [14, 21]. These two proteins are modified by HAUbVS and HAUbVME and are most likely responsible for the observed copurification of 19S subunits in our preparations. Interestingly, 19S subunits do not copurify with enzymes modified by HAUbBr2, suggesting that the 11 DUBs modified by this HAUb-derived probe do not associate in a stable manner with the 19S cap. Our data suggest that modified DUBs do not stably associate with other multisubunit complexes such as the COP9 signalosome, which shares homology with the 19S cap [4]. Recent reports have demonstrated that 19S proteins play a nonproteolytic role in nucleotide excision repair and transcription elongation [30]. In vivo, the base of the 19S complex can function independently of the larger complex and is implicated in RNA polymerase II-dependent transcription [31]. This may explain why many RNA binding proteins were isolated under native conditions (Table 2). Additionally, a recent study suggests a role for USP8 (UbpY) and USP4 (UNP) in the regulation of short-lived mRNA degradation [32]. DUBs could therefore play a regulatory role in transcription and RNA metabolism processes.

Significance

Deubiquitinating enzymes are emerging as a new class of regulators in the ubiquitin-proteasome system. The chemistry-based proteomics approach presented in this study allows isolation, identification, and demonstration of enzyme activity using specific probes against DUBs. We not only find novel enzymes of the DUB family, but also demonstrate DUB-like enzymatic activity of a novel putative superfamily of proteases. Labeling profiles obtained with different thiol-reactive groups demonstrates that the reactivity of these probes can be fine-tuned, which opens the way for designing inhibitors that could target individual enzymes more selectively. The intein-based method facilitates the coupling of a variety of chemical moieties onto the C terminus of Ub and potentially other proteins. Additionally, HAUb-derived probes with broad reactivity, such as HAUbVME, can be used to rapidly screen the activity of many DUBs in cells subjected to different experimental conditions or in tissue samples from healthy or diseased individuals. More generally, their use allows the unambiguous assignment of particular enzymatic function to open reading frames.

Experimental Procedures

Methanesulfonylmethyl-phosphonic acid diethyl ester and benzenesulfonylmethyl-phosphonic acid diethyl ester were synthesized according to literature procedures [18, 19, 33] (see Supplemental Data for synthesis details). Slide-a-lyzer dialysis membranes were from Pierce. NMR spectra were recorded on a Varian 200 MHz spectrometer; mass spectra were recorded on an electrospray LCZ LC-MS instrument (LC HP1100 Hewlett Packert, MS Micromass, UK) equipped with a Waters DeltaPak C4 (3.9x150 mm) column.

Plasmid Construction

pTYB-HAUb plasmid was constructed by cloning the sequence of human Ub (lacking Gly76) into the pTYB2 vector (New England Biolabs) to generate an in-frame fusion with the intein and chitin binding domain. The HA tag was introduced by inserting an oligonucleotide cassette into the NdeI site at the 5′end of the Ub sequence.

Synthesis of $HAUb_{75}$-MESNa

Ub-intein-chitin domain fusion protein was expressed in *E. coli* (2 hr induction with 0.5 mM IPTG at 30°C). Cells pellets were resuspended in 50 ml 50 mM HEPES pH 6.5, 100 mM NaOAc, 50 μM PMSF and lysed by french press (1500 psi). The clarified cell extract was loaded onto a 15 ml chitin bead (New England Biolabs) column at a flow rate of 0.5 ml/min. The column was washed with 60 ml of lysis buffer followed by 25 ml of lysis buffer containing 50 mM β-mercaptoethanesulfonic acid sodium salt (MESNa) and incubated overnight at 37°C for the induction of on-column cleavage. $HAUb_{75}$-MESNa thioester was eluted with 25 ml of lysis buffer and concentrated: approximately 2.5 mg of protein was recovered from a 1L culture. The N-terminal Met of the HA-tag was frequently processed off, giving a mixture of two proteins that behaved identically in labeling experiments.

Synthesis and Purification of HAUb-Derived Active Site Thiol-Reactive Probes

HAUbCl, HAUbBr2, HAUbBr3

To a solution of $HAUb_{75}$-MESNa (1-2 mg/mL) in column buffer (500 μl) was added 0.2 mmol of the desired haloalkylamine haloacid salt and 100 μl of 2.0 M aqueous NaOH and the mixture was immediately vortexed. After 20 min at room temperature. 100 μl of 2.0 M aqueous HCl was added and the solution was dialyzed against 50 mM NaOAc (pH 4.5) in a 3.5 ml Pierce Slide-a-lyzer cassette (3500 MWCO) for 2 hr. The resulting product (>90% conversion estimated from LC-MS) was divided into aliquots and stored at −80°C (no significant deterioration is observed for several months of storage except for HAUbBr2, which is more unstable).

HAUbVME, HAUbVS, HAUbVCN

To a solution of $HAUb_{75}$-MESNa (1-2 mg/ml, 500 μl) was added 0.125 mmol of the desired Michael acceptor as *para*-toluene sulfonic acid salt followed by 75 μl of 2M *N*-hydroxy succinimide and 125 μl 2 M NaOH. The mixture was incubated at 37°C for 2 hr and reaction progress was monitored by LC-MS to give the desired products with 50%–60% conversion. The reaction mixture was neutralized by the addition of 125 μl of 2 M HCl and dialyzed as described above. HAUbVSPh and HAUb were synthesized similarly (see Supplemental Data).

All HAUb-derived probes were purified to 95% purity with the use of a Pharmacia SMART system MonoS 1.6/5 column, with a linear gradient from 0% to 30% B, 50 mM NaOAc (pH 4.5, buffer A), 50 mM NaOAc (pH 4.5), and 1 M NaCl (buffer B); HAUbVSPh gave a different elution profile because of the hydrophobicity of its C terminus. All synthetically modified HA-tagged ubiquitin derivatives were purified, except HAUbBr2, which is less stable. HAUbBr2 was used directly after dialysis (synthesis yield was >90%). All HAUb-derived probes were analyzed by LC-MS (ESI) using a C4 RP-HPLC column with a 0%–80% gradient over 20 min in a 0.1% formic acid/acetonitrile buffer system (see Supplemental Data for MS values).

Preparation of EL4 Cell Extracts and Labeling with HAUb Derivatives

EL-4 cells (cultured in RPMI-HEPES supplemented with 10% FCS, 1% glutamine, and 1% penicilline/streptomycine) were harvested and washed three times with PBS. Cell pellets were lysed with glass beads in buffer HR (50 mM Tris [pH 7.4], 5 mM $MgCl_2$, 250 mM sucrose, 1 mM DTT, 2 mM ATP). Nuclei were removed by centrifugation and 20 μg of protein extract was used for labeling with HAUb derivatives. Indicated concentrations of HAUb derivatives were incubated with cell extracts for 1 hr at 37°C. Immunoblots with the anti-HA monoclonal antibody 12CA5 were carried out according to standard protocols. SDS-PAGE gels (8%) were used to resolve high molecular weight DUBs.

Anti-HA Immunoprecipitation for Tandem Mass Spectrometry Analysis.

EL4 cell lysates were prepared as above, except 0.5–2 × 10^9 cells were used and 50 μM PMSF was included in the lysis buffer. Lysates (at 5 mg/ml) were incubated with the desired HAUb-derived probe (5 mg lysate and 6.6 μg of the probe for silver stains, 14–20 mg lysate and 20 μg of probe for Coomassie stains) for 2 hr at 37°C. SDS was added to "denatured" samples to the final concentration of 0.4% and then diluted to less then 0.1% with NET buffer (50 mM Tris [pH 7.5], 5 mM EDTA, 150 mM NaCl, 0.5% NP40) prior to the addition of anti-HA agarose. Anti-HA agarose (Sigma) was incubated with the samples overnight at 4°C, the immunoprecipitations were washed extensively with NET buffer, bound proteins were eluted with 50 mM glycine (pH 2.5) at 4°C for 30 min. All samples were evaporated to dryness and redisolved in 50 μl of 1× SDS-PAGE sample buffer. pH was adjusted with 1M Tris (pH 8) if needed. Samples were resolved by 8% reducing SDS-PAGE and stained with silver or Coommassie stain using standard conditions [34, 35].

Protein Identification by Tandem Mass Spectrometry

Individual polypeptides were excised from gels, destained, and subjected to trypsinolysis [36]. The samples were separated using a nanoflow liquid chromatrography system (Waters Cap LC) equipped with a picofrit column (75micron ID, 10cm, NewObjective) at a flow rate of approximately 150 nl/min using a nanotee (Waters Cap LC) 16/1 split (initial flow rate 5.5μl/min). The LC system was directly coupled to a tandem mass spectrometer (Q-TOF micro, Micromass). Analysis was performed in survey scan mode and parent ions with intensities greater than 6 were sequenced in MS/MS mode using MassLynx 3.5 Software (Micromass). MS/MS data were processed and subjected to database searches using ProteinLynx Global Server 1.1 Software (Micromass) against Swissprot, TREMBL/New (http://www.expasy.ch), or using Mascot (Matrixscience) against the NCBI non-redundant (nr) or mouse EST databases.

Supplemental Data

Additional methods and mass spectrometry data on the HAUb-derived probes are provided in the Supplemental Material that accompanies this paper. Please write to chembiol@cell.com for a PDF.

Acknowledgments

We thank LeAnn Williams for expert preparation of samples for MS/MS analysis. This work is supported by National Institutes of Health Grants 1RO1 GM62502 (H.L.P.), 1R01 GM30308 and GM066355 (K.D.W.), and TW05461-01 (T.G.E.). H.O. is financially supported by a fellowship from the Netherlands Organization for Scientific Research (NWO).

Received: July 31, 2002
Revised: September 4, 2002
Accepted: September 5, 2002

References

1. Gewurz, B.E., Gaudet, R., Tortorella, D., Wang, E.W., and Ploegh, H.L. (2001). Virus subversion of immunity: a structural perspective. Curr. Opin. Immunol. *13*, 442–450.
2. Greenbaum, D., Medzihradszky, K.F., Burlingame, A., and Bogyo, M. (2000). Epoxide electrophiles as activity-dependent cysteine protease profiling and discovery tools. Chem. Biol. *7*, 569–581.
3. Kidd, D., Liu, Y., and Cravatt, B.F. (2001). Profiling serine hydrolase activities in complex proteomes. Biochemistry *40*, 4005–4015.
4. Glickman, M.H., and Ciechanover, A. (2002). The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol. Rev. *82*, 373–428.
5. Pickart, C.M. (2001). Mechanisms underlying ubiquitination. Annu. Rev. Biochem. *70*, 503–533.
6. Wilkinson, K.D. (2000). Ubiquitination and deubiquitination: targeting of proteins for degradation by the proteasome. Semin. Cell Dev. Biol. *11*, 141–148.
7. Li, M., Chen, D., Shiloh, A., Luo, J., Nikolaev, A.Y., Qin, J., and Gu, W. (2002). Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization. Nature *416*, 648–653.
8. Bignell, G.R., Warren, W., Seal, S., Takahashi, M., Rapley, E., Barfoot, R., Green, H., Brown, C., Biggs, P.J., Lakhani, S.R., et al. (2000). Identification of the familial cylindromatosis tumour-suppressor gene. Nat. Genet. *25*, 160–165.
9. Jensen, D.E., Proctor, M., Marquis, S.T., Gardner, H.P., Ha, S.I., Chodosh, L.A., Ishov, A.M., Tommerup, N., Vissing, H., Sekido, Y., et al. (1998). BAP1: a novel ubiquitin hydrolase which binds to the BRCA1 RING finger and enhances BRCA1-mediated cell growth suppression. Oncogene *16*, 1097–1112.
10. Nakamura, T., Hillova, J., Mariage-Samson, R., Onno, M., Huebner, K., Cannizzaro, L.A., Boghosian-Sell, L., Croce, C.M., and Hill, M. (1992). A novel transcriptional unit of the tre oncogene widely expressed in human cancer cells. Oncogene *7*, 733–741.
11. Mimnaugh, E.G., Kayastha, G., McGovern, N.B., Hwang, S.G., Marcu, M.G., Trepel, J., Cai, S.Y., Marchesi, V.T., and Neckers, L. (2001). Caspase-dependent deubiquitination of monoubiquitinated nucleosomal histone H2A induced by diverse apoptogenic stimuli. Cell Death Differ. *8*, 1182–1196.
12. Raboy, B., Parag, H.A., and Kulka, R.G. (1986). Conjugation of [125I]ubiquitin to cellular proteins in permeabilized mammalian cells: comparison of mitotic and interphase cells. EMBO J. *5*, 863–869.
13. Dupre, S., and Haguenauer-Tsapis, R. (2001). Deubiquitination step in the endocytic pathway of yeast plasma membrane proteins: crucial role of Doa4p ubiquitin isopeptidase. Mol. Cell. Biol. *21*, 4482–4494.
14. Borodovsky, A., Kessler, B.M., Casagrande, R., Overkleeft, H.S., Wilkinson, K.D., and Ploegh, H.L. (2001). A novel active site-directed probe specific for deubiquitylating enzymes reveals proteasome association of USP14. EMBO J. *20*, 5187–5196.
15. Cotton, G.J., and Muir, T.W. (1999). Peptide ligation and its application to protein engineering. Chem. Biol. *6*, R247–R256.
16. Chong, S., Mersha, F.B., Comb, D.G., Scott, M.E., Landry, D., Vence, L.M., Perler, F.B., Benner, J., Kucera, R.B., Hirvonen, C.A., et al. (1997). Single-column purification of free recombinant proteins using a self- cleavable affinity tag derived from a protein splicing element. Gene *192*, 271–281.
17. Makarova, K.S., Aravind, L., and Koonin, E.V. (2000). A novel superfamily of predicted cysteine proteases from eukaryotes, viruses and Chlamydia pneumoniae. Trends Biochem. Sci. *25*, 50–52.
18. Liu, S., and Hanzlik, R.P. (1992). Structure-activity relationships

for inhibition of papain by peptide Michael acceptors. J. Med. Chem. *35*, 1067–1075.

19. Dragovich, P.S., Webber, S.E., Babine, R.E., Fuhrman, S.A., Patick, A.K., Matthews, D.A., Lee, C.A., Reich, S.H., Prins, T.J., Marakovits, J.T., et al. (1998). Structure-based design, synthesis, and biological evaluation of irreversible human rhinovirus 3C protease inhibitors. 1. Michael acceptor structure-activity studies. J. Med. Chem. *41*, 2806–2818.
20. Larsen, C.N., Price, J.S., and Wilkinson, K.D. (1996). Substrate binding and catalysis by ubiquitin C-terminal hydrolases: identification of two active site residues. Biochemistry *35*, 6735–6744.
21. Lam, Y.A., Xu, W., DeMartino, G.N., and Cohen, R.E. (1997). Editing of ubiquitin conjugates by an isopeptidase in the 26S proteasome. Nature *385*, 737–740.
22. Holzl, H., Kapelari, B., Kellermann, J., Seemuller, E., Sumegi, M., Udvardy, A., Medalia, O., Sperling, J., Muller, S.A., Engel, A., et al. (2000). The regulatory complex of Drosophila melanogaster 26S proteasomes. Subunit composition and localization of a deubiquitylating enzyme. J. Cell Biol. *150*, 119–130.
23. Schevchenko, A., Schaft, D., Roguev, A., Pijnappel, W.W., Stewart, A.F., and Schevchenko, A. (2002). Deciphering protein complexes and protein interaction networks by tandem affinity purification and mass spectrometry. Mol. Cell. Proteomics *1*, 204–212.
24. Amerik, A.Y., Li, S.J., and Hochstrasser, M. (2000). Analysis of the deubiquitinating enzymes of the yeast Saccharomyces cerevisiae. Biol. Chem. *381*, 981–992.
25. Hershko, A., and Rose, I.A. (1987). Ubiquitin-aldehyde: a general inhibitor of ubiquitin-recycling processes. Proc. Natl. Acad. Sci. USA *84*, 1829–1833.
26. Malakhov, M.P., Malakhova, O.A., Kim, K.I., Ritchie, K.J., and Zhang, D.E. (2002). UBP43 (USP18) specifically removes ISG15 from conjugated proteins. J. Biol. Chem. *277*, 9976–9981.
27. Steinhauer, W.R., and Kalfayan, L.J. (1992). A specific ovarian tumor protein isoform is required for efficient differentiation of germ cells in Drosophila oogenesis. Genes Dev. *6*, 233–243.
28. Scheffner, M., Nuber, U., and Huibregtse, J.M. (1995). Protein ubiquitination involving an E1–E2–E3 enzyme ubiquitin thioester cascade. Nature *373*, 81–83.
29. Voges, D., Zwickl, P., and Baumeister, W. (1999). The 26S proteasome: a molecular machine designed for controlled proteolysis. Annu. Rev. Biochem. *68*, 1015–1068.
30. Ferdous, A., Gonzalez, F., Sun, L., Kodadek, T., and Johnston, S.A. (2001). The 19S regulatory particle of the proteasome is required for efficient transcription elongation by RNA polymerase II. Mol. Cell *7*, 981–991.
31. Gonzalez, F., Delahodde, A., Kodadek, T., and Johnston, S.A. (2002). Recruitment of a 19S proteasome subcomplex to an activated promoter. Science *296*, 548–550.
32. Laroia, G., Sarkar, B., and Schneider, R.J. (2002). Ubiquitin-dependent mechanism regulates rapid turnover of AU-rich cytokine mRNAs. Proc. Natl. Acad. Sci. USA *99*, 1842–1846.
33. Bogyo, M., Shin, S., McMaster, J.S., and Ploegh, H.L. (1998). Substrate binding and sequence preference of the proteasome revealed by active-site-directed affinity probes. Chem. Biol. *5*, 307–320.
34. DeSilva, T.M., Ursitti, J., and Speicher, D.W. (1995). Protein detection in gels using fixation. In Current Protocols in Protein Science, J.E. Coligan, et al., eds. (New York: John Wiley & Sons), pp. 10.5.1–10.5.11.
35. Mortz, E., Krogh, T.N., Vorum, H., and Gorg, A. (2001). Improved silver staining protocols for high sensitivity protein identification using matrix-assisted laser desorption/ionization-time of flight analysis. Proteomics *1*, 1359–1363.
36. Kinter, M., and Sherman, N.E. (2000). Protein Sequencing and Identification Using Tandem Mass Spectrometry (New York: Wiley & Sons).
37. Saitou, N., and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. *4*, 406–425.
38. Blanchette, P., Gilchrist, C.A., Baker, R.T., and Gray, D.A. (2001). Association of UNP, a ubiquitin-specific protease, with the pocket proteins pRb, p107 and p130. Oncogene *20*, 5533–5537.
39. Wilkinson, K.D., Tashayev, V.L., O'Connor, L.B., Larsen, C.N., Kasperek, E., and Pickart, C.M. (1995). Metabolism of the polyubiquitin degradation signal: structure, mechanism, and role of isopeptidase T. Biochemistry *34*, 14535–14546.
40. Everett, R.D., Meredith, M., Orr, A., Cross, A., Kathoria, M., and Parkinson, J. (1997). A novel ubiquitin-specific protease is dynamically associated with the PML nuclear domain and binds to a herpesvirus regulatory protein. EMBO J. *16*, 1519–1530.
41. Zapata, J.M., Pawlowski, K., Haas, E., Ware, C.F., Godzik, A., and Reed, J.C. (2001). A diverse family of proteins containing tumor necrosis factor receptor- associated factor domains. J. Biol. Chem. *276*, 24242–24252.
42. Naviglio, S., Mattecucci, C., Matoskova, B., Nagase, T., Nomura, N., Di Fiore, P.P., and Draetta, G.F. (1998). UBPY: a growth-regulated human ubiquitin isopeptidase. EMBO J. *17*, 3241–3250.
43. Gnesutta, N., Ceriani, M., Innocenti, M., Mauri, I., Zippel, R., Sturani, E., Borgonovo, B., Berruti, G., and Martegani, E. (2001). Cloning and characterization of mouse UBPy, a deubiquitinating enzyme that interacts with the ras guanine nucleotide exchange factor CDC25(Mm)/Ras-GRF1. J. Biol. Chem. *276*, 39448–39454.
44. Kato, M., Miyazawa, K., and Kitamura, N. (2000). A deubiquitinating enzyme UBPY interacts with the Src homology 3 domain of Hrs-binding protein via a novel binding motif PX(V/I)(D/N)RXXKP. J. Biol. Chem. *275*, 37481–37487.
45. Chen, X., Zhang, B., and Fischer, J.A. (2002). A specific protein substrate for a deubiquitinating enzyme: Liquid facets is the substrate of Fat facets. Genes Dev. *16*, 289–294.
46. Taya, S., Yamamoto, T., Kanai-Azuma, M., Wood, S.A., and Kaibuchi, K. (1999). The deubiquitinating enzyme Fam interacts with and stabilizes beta- catenin. Genes Cells *4*, 757–767.
47. Soncini, C., Berdo, I., and Draetta, G. (2001). Ras-GAP SH3 domain binding protein (G3BP) is a modulator of USP10, a novel human ubiquitin specific protease. Oncogene *20*, 3869–3879.
48. Ideguchi, H., Ueda, A., Tanaka, M., Yang, J., Tsuji, T., Ohno, S., Hagiwara, E., Aoki, A., and Ishigatsubo, Y. (2002). Structural and functional characterization of the USP11 deubiquitinating enzyme, which interacts with the RanGTP-associated protein RanBPM. Biochem. J. *367*, 87–95.
49. Timms, K.M., Ansari-Lari, M.A., Morris, W., Brown, S.N., and Gibbs, R.A. (1998). The genomic organization of Isopeptidase T-3 (ISOT-3), a new member of the ubiquitin specific protease family (UBP). Gene *217*, 101–106.
50. Verma, R., Chen, S., Feldman, R., Schieltz, D., Yates, J., Dohmen, J., and Deshaies, R.J. (2000). Proteasomal proteomics: identification of nucleotide-sensitive proteasome-interacting proteins by mass spectrometric analysis of affinity-purified proteasomes. Mol. Biol. Cell *11*, 3425–3439.
51. Baker, R.T., Wang, X.W., Woollatt, E., White, J.A., and Sutherland, G.R. (1999). Identification, functional characterization, and chromosomal localization of USP15, a novel human ubiquitin-specific protease related to the UNP oncoprotein, and a systematic nomenclature for human ubiquitin-specific proteases. Genomics *59*, 264–274.
52. Cai, S.Y., Babbitt, R.W., and Marchesi, V.T. (1999). A mutant deubiquitinating enzyme (Ubp-M) associates with mitotic chromosomes and blocks cell division. Proc. Natl. Acad. Sci. USA *96*, 2828–2833.
53. Henchoz, S., De Rubertis, F., Pauli, D., and Spierer, P. (1996). The dose of a putative ubiquitin-specific protease affects position- effect variegation in Drosophila melanogaster. Mol. Cell. Biol. *16*, 5717–5725.
54. Saigoh, K., Wang, Y.L., Suh, J.G., Yamanishi, T., Sakai, Y., Kiyosawa, H., Harada, T., Ichihara, N., Wakana, S., Kikuchi, T., et al. (1999). Intragenic deletion in the gene encoding ubiquitin carboxy-terminal hydrolase in gad mice. Nat. Genet. *23*, 47–51.
55. Larsen, C.N., Krantz, B.A., and Wilkinson, K.D. (1998). Substrate specificity of deubiquitinating enzymes: ubiquitin C-terminal hydrolases. Biochemistry *37*, 3358–3368.
56. Kurihara, L.J., Semenova, E., Levorse, J.M., and Tilghman, S.M. (2000). Expression and functional analysis of Uch-L3 during mouse development. Mol. Cell. Biol. *20*, 2498–2504.
57. Gilchrist, C.A., Gray, D.A., and Baker, R.T. (1997). A ubiquitin-specific protease that efficiently cleaves the ubiquitin- proline bond. J. Biol. Chem. *272*, 32280–32285.

58. Layfield, R., Franklin, K., Landon, M., Walker, G., Wang, P., Ramage, R., Brown, A., Love, S., Urquhart, K., Muir, T., et al. (1999). Chemically synthesized ubiquitin extension proteins detect distinct catalytic capacities of deubiquitinating enzymes. Anal. Biochem. *274*, 40–49.
59. Hansen-Hagge, T.E., Janssen, J.W., Hameister, H., Papa, F.R., Zechner, U., Seriu, T., Jauch, A., Becke, D., Hochstrasser, M., and Bartram, C.R. (1998). An evolutionarily conserved gene on human chromosome 5q33-q34, UBH1, encodes a novel deubiquitinating enzyme. Genomics *49*, 411–418.
60. Yin, L., Krantz, B., Russell, N.S., Deshpande, S., and Wilkinson, K.D. (2000). Nonhydrolyzable diubiquitin analogues are inhibitors of ubiquitin conjugation and deconjugation. Biochemistry *39*, 10001–10010.
61. Mayer, A.N., and Wilkinson, K.D. (1989). Detection, resolution, and nomenclature of multiple ubiquitin carboxyl-terminal esterases from bovine calf thymus. Biochemistry *28*, 166–172.