Identification and Characterization of DEN1, a Deneddylase of the ULP Family*

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To identify deneddyllases, proteases with specificity for hydrolysis of Nedd8 derivatives, a facile method was developed for the synthesis of Nedd8 amidomethylcoumarin (a substrate) and Nedd8 vinyl sulfone (an inhibitor). Deneddylase activity is necessary to reverse the conjugation of Nedd8 to cullin, a modification that regulates at least some ubiquitin ligases. The reaction of Nedd8 vinyl sulfone with L-M(TM) mouse fibroblast lysates identified two deneddyllases. The deubiquitinating enzyme UCH-L3 is labeled by both ubiquitin vinyl sulfone and Nedd8 vinyl sulfone. In contrast, a second and more selective enzyme is labeled only by Nedd8 vinyl sulfone. This protein, DEN1, is a 221-amino acid thiol protease that is encoded by an open reading frame previously annotated as SENP8. Recombinant human DEN1 shows significant specificity for Nedd8 and catalyzes the hydrolysis of Nedd8 amidomethylcoumarin with a 

Ubiquitin (Ub)¹ and the ubiquitin-like proteins (Ubl) constitute a family of proteins that are post-translationally conjugated to other proteins to target them for specific localization (1–4). Thus, attachment of a Lys⁴⁸-linked polyubiquitin chain targets proteins to the proteasome, the multicatalytic protease that is responsible for most regulated intracellular proteolysis (5, 6). Ubiquitination is also involved in numerous other processes, including DNA repair, establishment, and maintenance of chromatin structure, receptor internalization, and small or disordered from the C terminus of ubiquitin (17). Several enzymes exemplified by the RPN11 subunit of the proteasome and the CSN5 subunit of the COP9 signalosome. There probably are others.

Several ubiquitin-like proteins are also conjugated to other proteins and likewise act as targeting signals for the attached proteins (8, 9). Nedd8 is about 50% identical to ubiquitin and its conjugation to the cullin component of several ubiquitin ligases is thought to increase the efficiency of polyubiquitination (4). SUMO-1, -2, and -3 are closely related proteins with about 20% identity to ubiquitin that are attached to numerous proteins in the nucleus and to septins that accumulate at the bud neck of yeast (2, 10–12). Whereas the precise sites of localization and the mechanisms by which it is accomplished are not known, several important cellular regulators are sumoylated. These include p53, proliferating cell nuclear antigen, topoisomerase II, promyelocytic leukemia protein, and IκBα (2, 10–12). Finally, ISG15 is an interferon-stimulated protein with two ubiquitin-like domains that is attached to a poorly characterized set of proteins and results in a cytoskeletal distribution of ISG15 conjugates (3, 13, 14).

Modification by ubiquitin-like proteins is reversible. The removal of the ubiquitin domain is catalyzed by processing proteases that have been generally named deubiquitinating (DUB)² enzymes (15, 16). At least four gene families are well defined. Ubiquitin C-terminal hydrolases (UCH) are thiol proteases with a 230-amino acid core catalytic domain and that show specificity for hydrolysis of leaving groups that are either small or disordered from the C terminus of ubiquitin (17). These enzymes exhibit tight binding of ubiquitin derivatives but will also process small derivatives of Nedd8 (18). There is

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¹ The abbreviations used are: Ub, ubiquitin; Ubl, ubiquitin-like proteins; such as Nedd8, SUMO-1, ISG15, etc.; DUB, deubiquitinating; AMC, 7-amino-4-methylcoumarin; Ub-VS, ubiquitin C-terminal vinyl sulfone; Nedd8-VS, Nedd8 C-terminal vinyl sulfone; MESNA, mercaptotethane sulfonic acid; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; UCH, ubiquitin C-terminal hydrolases; ULP, ubiquitin-like proteases.

‡ Nomenclature used is as follows. Deubiquitinating enzyme (DUB) is a general term for any protease with specificity for the hydrolysis of amide or ester bonds at the C terminus of Ub or Ubl. There are four well defined gene families that encode DUBs: UCH, ubiquitin C-terminal hydrolase (Pfam motif PF01088); UBP/USP, ubiquitin-specific proteases (Pfam motif PF00443); ULP, ubiquitin-like protease (Pfam motif PF00290); and JAMM isopeptidases, the Jab1/MPN domain metalloenzymes exemplified by the RPN11 subunit of the proteasome and the CSN5 subunit of the COP9 signalosome. There probably are others. DUBs can be given descriptive names based on the reaction catalyzed by these DUBs. Thus, an enzyme that is specific for ubiquitin is designated as a deubiquitinase, one specific for hydrolysis of Nedd8 derivatives is a deneddyllase, etc.

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I and inserted into pTYB2 (New England Biolabs) digested with NdeI and another to hydrolyze Nedd8 derivatives (21). Ubiquitin-like proteases (ULP) remove SUMOs from other proteins, although the exact specificities are ill defined (22, 23). There are two such enzymes in yeast and seven in man. Finally, JAMM isopeptidases (24–26) have been shown to deneddylate cullins (the CSN-5 subunit of the COP9/signalosome) and disassemble polyubiquitin chains (the RPN11 subunit of the proteasome).

The presence of so many DUBs suggests multiple roles for deubiquitination. These include processing of the Ubp protein, reversal of ubiquitination to down-regulate localization processes, and disassembly of polyubiquitin chains to recycle ubiquitin. DUBs have been shown to be oncogenes, to modulate gene silencing and chromatin structure, and to affect developmental processes such as neuronal migration and survival, chemotaxis, differentiation, and eye development (15, 16). One of the outstanding challenges in this field is to define and understand substrate specificity and the physiological roles of these important modulators of the ubiquitin pathway.

The only known substrates for Nedd8 conjugation (neddylation) are the cullins (27). Cullins are subunits of many ubiquitin ligases. These multimeric ligases are widespread and their substrate specificity is probably determined by the specific combination of cullins and adapter proteins in the assembled ligase. Optimal activity of these ligases requires neddylation of the cullin component (4, 28, 29). This modification is thought to assist in recruiting the ubiquitin-E2 thiol ester to the neddylation site. Nedd8-specific DUBs (deneddylases) are required to process the primary gene product by removal of the C-terminal propeptide, as well as to remove Nedd8 from the cullins. Normally, cullin modification is limited to a single Nedd8, although cullins can be hyper-neddylated in vitro. The Deshaies group (26) reported that mutations in the COP9 signalosome result in the accumulation of singly neddylated cullins. This suggests that the cullin-containing E3 ligases may be regulated by the neddylation activity of the signalosome. Processing of the propeptide of Nedd8 is less well defined, but may be accomplished by UCH enzymes (30).

The current work focuses on defining the DUBs that catalyze deneddylation. Using a technique we developed for identification of the corresponding ubiquitin-specific enzymes (31, 32) we have synthesized the C-terminal vinyl sulfone of Nedd8 (Nedd8-VS). This reactive vinyl group is subject to Michael addition and Nedd8-VS is an active site-directed irreversible inhibitor of deneddylases. The addition of an epitope tag to this inhibitor allowed us to purify and identify two deneddylases from L-M(TK\textsuperscript{C}) mouse fibroblasts. The same approach can be utilized to synthesize Nedd8-AMC, the C-terminal amidomethylcoumarin of Nedd8, for use as a substrate. The results demonstrate that the DEN1 deneddylase is a member of the ULP family with exquisite specificity for Nedd8.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Protein Expression—A cDNA encoding residues 1–75 of human Nedd8 was amplified from pRSNedd8(G2) by PCR using the primers 5′-CTTACAGGCGCAACACCG-3′ and 5′-TCTTCAGGCGCAACACCG-3′. The PCR product was digested with NdeI and inserted into pTYB2 (New England Biolabs) digested with NdeI and Smal prior to ligat...
RESULTS

New Nedd8-specific Inhibitors and Substrates—Identification of deneddylases will be facilitated by the availability of specific inhibitors and substrates. To synthesize inhibitors and substrates of the Ubl protein processing proteases we have adapted the intein fusion protein approach (35) to produce ubiquitin-like proteins activated at their C terminus. In this method, the ubiquitin-like protein, lacking its C-terminal glycine, is produced in bacteria as a fusion protein with a self-splicing intein followed by a chitin binding domain (Fig. 1). Expressed fusion protein was adsorbed to a chitin column and contaminating proteins were removed by washing. Nedd8-75 (or FLAG-Nedd8-75) was isolated as the C-terminal thiol ester by incubating the column with MESNA. Cleavage from the column is quantitative and the yield of homogeneous Nedd8-75 thiol ester (expected mass 8626.13, observed mass 8626.92) was 3 mg/liter of bacterial culture. Reaction of this thiol ester with nucleophiles (in the presence of N-hydroxysuccinimide as a catalyst) leads to a variety of C-terminal derivatives. For instance, reaction of the MESNA ester of FLAG-Nedd8-75 thiol ester with glycyl vinylmethyl sulfone (32) resulted in the synthesis of FLAG-Nedd8-AMC. After purification by ion exchange chromatography, the yield was about 30% (mass expected 9997.50, mass observed 9997.25).

The results above also suggested that we could synthesize substrates of Nedd8-specific processing proteases (deneddylases) using the same chemistry. We chose to synthesize Nedd8-AMC, a substrate whose cleavage should result in the release of the intensely fluorescent AMC group (36). To increase the solubility of the nucleophillic coumarin, we took advantage of the fact that cyclodextrins increase the solubility of many hydrophobic compounds. Reaction of Nedd8-75 thiol ester with glycyl 7-amido-4-methylcoumarin (50 mM) in the presence of 30% hydroxypropyl-β-cyclodextrin resulted in the synthesis of C-terminal amidomethylcoumarin of Nedd8 (Nedd8-AMC) in about 5% yield. After purification on ion exchange chromatography, Nedd8-AMC was shown to exhibit the expected mass (Fig. 1), fluorescence spectra, and properties as a substrate (Fig. 2).

Because the deubiquitinating enzyme UCH-L3 catalyzes the hydrolysis of Nedd8 derivatives we tested if UCH-L3 would hydrolyze Nedd8-AMC. As expected, Nedd8-AMC was completely and efficiently hydrolyzed by UCH-L3 (Fig. 2). When added to this reaction, FLAG-Nedd8-AMC was an efficient and irreversible inhibitor of the hydrolysis of Nedd8-AMC by UCH-L3 (Fig. 2). This inhibition was complete, even when the more tightly bound substrate Ub-AMC was used, suggesting that the vinyl sulfone covalently modifies the enzyme. Thus, each of these derivatives was synthesized in good yield and possessed the expected specificity with UCH-L3, a known deneddylase.

Detection of Deneddylases in Mammalian Cell Extracts—To detect and identify deneddylases in mammalian cells, we reacted a lysate of L-MTK mouse fibroblasts with FLAG-Nedd8-AMC. Based on similar experiments with Ub-AMC (31, 32), we expected that only deneddylases would be rapidly and specifically labeled by this reagent. Fig. 3A shows that when this reaction mixture is subjected to SDS-PAGE and Western blotting with anti-FLAG antibodies two proteins are labeled. A major protein reacts to give an adduct with an apparent molecular weight of about 40,000 and a minor protein yields an adduct of apparent molecular weight of about 35,000. Similar results were obtained with lysates of COS and HeLa cells, although the Western blots showed that more cross-reactive...
background bands were present in the unmodified lysates (data not shown). As these bands would complicate purification of the authentic deneddylases we conducted the rest of our studies with L-M(TK-)
lysates.

To identify the proteins labeled by FLAG-Nedd8-VS we immunopurified the adducts formed by adsorbing the reaction mixture to anti-FLAG M2-Sepharose. Fig. 3A shows that immunoreactive FLAG-Nedd8 is completely adsorbed by the column and can be eluted with FLAG peptide. Purification of FLAG-Nedd8-VS-labeled proteins was monitored by 4–20% gradient SDS-PAGE and Western blotting analysis with anti-FLAG M5 antibody. The column was regenerated with a low pH wash. β-Actin was present in control reactions lacking FLAG-Nedd8-VS. B, cell lysate was preblocked with Ub-VS then labeled with FLAG-Nedd8-VS. The incorporation of FLAG-Nedd8 into Nedd8-specific proteases was monitored by 4–20% gradient SDS-PAGE Western blotting analysis. Prelabeling blocked the reactivity of UCH-L3 and revealed a new Nedd8-specific protease we called DEN1.

To investigate the specificity of these labeling experiments, we first prelabeled the lysates with Ub-VS (to block any Ub-specific enzymes) and then added FLAG-Nedd8-VS to derivatize any Nedd8-specific enzymes (Fig. 3B). In this experiment, control Western blots showed two weak immunoreactive bands near, but not identical to, the observed adducts (*) in Fig. 3B. Labeling with FLAG-Nedd8-VS alone yielded the pattern seen previously. However, reaction of the prelabeled lysates with FLAG-Nedd8-VS revealed an almost complete loss of adduct corresponding to UCH-L3 but persistence of the labeled band at 35 kDa. Increasing the concentration of FLAG-Nedd8-VS 10-fold did not result in the labeling of any additional species. Thus, UCH-L3 reacts with both Ub-VS and FLAG-Nedd8-VS while the adduct at 35 kDa arises from a protein that only reacts with FLAG-Nedd8-VS. This latter reaction can be completely eliminated by incubation with Nedd8 (data not shown), suggesting that the FLAG and vinyl sulfone moieties are not contributing significantly to the specificity of labeling. We propose that the enzyme modified by FLAG-Nedd8-VS to give the 35-kDa band be called deneddylyase 1 (DEN1). Note that DEN1 is considerably less abundant than UCH-L3 in these lysates.

Identification of DEN1—To identify DEN1, we purified the protein with anti-FLAG M2-Sepharose as described above for UCH-L3. The proteins eluted from the column with the FLAG peptide were separated by SDS-PAGE and the portion of the gel containing the adduct was excised from the gel, digested...
with trypsin, and subjected to LC MS/MS analysis (Fig. 4). Several peptides derived from β-actin and β-actin-binding proteins (ARP2/3 protein complex 34, tropomodulin, and tropomyosin) were present indicating contamination with cytoskeletal proteins (Table I). As suggested above, these are probably non-specifically bound. Similarly, the ribosomal proteins L5, L3a, and nascent-polypeptide-associated complex α were identified, suggesting that some contamination with abundant ribosomes was also observed. However, none of these proteins are expected to be modified by FLAG-Nedd8-VS and it is assumed that they are trace contaminants. As expected, the band analyzed also contained 4 peptides from Nedd8, as well as five peptides from a previously identified ubiquitin-like protease family member annotated as SENP8 (GenBank™ accession number AAH37443) was the best candidate for SENP8. 

A cDNA encoding the 212-amino acid human SENP8 (GenBank™ accession number AAG21828) was obtained and recombinant protein was expressed in bacteria (see accompanying manuscript, Ref. 47). Purified recombinant SENP8 was used to raise antibodies in rabbits and to purify the anti-SENP8 antibody. When lysates of L-M(TK) cells were subjected to Western blotting with affinity purified anti-SENP8 a single band with an apparent molecular mass of ~28 kDa was observed (Fig. 5). This protein was unaffected by prelabeling the lysates with Ub-VS, but is quantitatively converted to a 35-kDa adduct upon reaction of the preblotted lysates with FLAG-Nedd8-VS. Likewise, this adduct was adsorbed by the anti-FLAG M2-Sepharose and eluted from the resin by FLAG peptide. Anti-FLAG reactivity and anti-SENP8 reactivity show a similar elution profile.

**Catalytic Properties of DEN1**—The results presented above suggest that DEN1 (SENP8) is a specific deneddylyase that has more affinity for, or reactivity with, FLAG-Nedd8-VS than it does with Ub-VS. Accordingly, recombinant human DEN1 was shown to react readily with FLAG-Nedd8-VS (Fig. 6). Quantitative analysis of this titration by SDS-PAGE shows that nearly 90% of the recombinant DEN1 was modified by equimolar FLAG-Nedd8-VS. Similar titrations with Ub-VS show no modification of DEN1 by this reagent under the same conditions. Thus, DEN1 shows marked specificity and appears to prefer Nedd8 as a substrate over ubiquitin.

We quantitated this preference by measuring the steady-state kinetics of DEN1 catalyzed hydrolysis of Nedd8-AMC, Ub-AMC, and SUMO-1-AMC. At 40 nM each substrate, the observed relative rates of hydrolysis by DEN1 were 60,000, 1, and <0.01, respectively. Thus, at low substrate concentrations the enzyme prefered Nedd8 as a substrate over the others by over 4 orders of magnitude. A full kinetic analysis of this reaction revealed that DEN1-catalyzed hydrolysis of Nedd8-AMC proceeded with a $K_m$ of 51 nM, a $k_{cat}$ of 7 s$^{-1}$, and a $k_{cat}/K_m$ of 1.3 × 10$^8$ M$^{-1}$ s$^{-1}$ (Table II). Because the $K_m$ for the DEN1-catalyzed hydrolysis of Ub-AMC was over 5 μM we could not determine the $K_m$ or $k_{cat}$, but $k_{cat}/K_m$ was 2.2 × 10$^3$ M$^{-1}$ s$^{-1}$. Thus, DEN1 is capable of hydrolyzing ubiquitin derivatives, but the selectivity (reflected in the $k_{cat}/K_m$ values) is markedly biased toward using Nedd8 as substrate. The true affinity of DEN1 for Ub and Nedd8 was estimated by measuring the $K_m$ for competitive inhibition of hydrolysis of Nedd8-AMC by the free Ubl domains. $K_m$ for Nedd8 was 185 μM while that for ubiquitin was so high that it could not be determined but must exceed 10 μM (Table II).

Table II also shows that the catalytic properties of UCH-L3 are exactly the converse. UCH-L3 shows very tight binding of Ub, its preferred substrate, with much weaker affinity for pro-Nedd8. In each optimal case (i.e. DEN1 acting upon Nedd8 and UCH-L3 acting on Ub) the reaction is rate limited by diffusion and catalysis itself is very fast. By all criteria DEN1 is a Nedd8 selective processing protease and appears to correspond to the previously identified open reading frame called SENP8.

**DISCUSSION**

Studies on Ub-specific DUBs have benefited greatly from the development of specific substrates and inhibitors. Ubiquitin ethyl ester was the first synthetic substrate described and proved useful in isolating and determining the enzymatic specificity of ubiquitin-C-terminal hydrolases (34, 37). More recently, ubiquitin-AMC has allowed the continuous spectrofluorometric characterization of other DUBs (36). Similarly, the inhibitors ubiquitin aldehyde and ubiquitin vinyl sulfone have been widely used as specific inhibitors and affinity reagents in studies of enzymatic specificity and enzyme structure (31, 32, 38–40). All these derivatives can be synthesized using the trypsin-catalyzed transpeptidation of ubiquitin first described over 15 years ago (34). Similar reagents have not been available for the corresponding processing proteases of the ubiquitin-like proteins, in part because of the fact that trypsin-catalyzed transpeptidation is not applicable with the other ubiquitin-like proteins. Our intein-based chemistry was developed specifically to make such reagents. The expression of several ubiquitin-like proteins as fusion proteins with the intein-chitin binding domain furnishes a facile route to the synthesis of these derivatives. Trapping of the thiol ester released upon intein-catalyzed cleavage with amines leads to the synthesis of a variety of useful C-terminal derivatives. The current work illustrates the utility of these reagents and has led to the identification of a new deneddylyase, one with a surprising specificity and potentially interesting role in the Nedd8 pathway.

**Nedd8-AMC Is a Good Substrate for Deneddylylases**—We at-
tempted to use the intein chemistry described here to synthesize Nedd8-AMC, but the solubility of the nucleophile glycyl-AMC is so low that little product was observed. Cyclodextrin has previously been shown to form a 2:2 host-guest complex with coumarins, resulting in increased solubility of the coumarin (41). The orientation of the coumarin in this complex is such that the $\beta$-amino group of glycyl-AMC protrudes into solvent and is available for reaction. Thus, addition of hydroxypropyl-cyclodextrin to the reaction resulted in a much higher amount of nucleophile and significantly improved yield of product. While the yield of Nedd8-AMC is low, it is severalfold higher than that obtained for Ub-AMC and the reaction can be scaled up to give useful amounts of substrate.

UCH and UBP/USP family members are efficient catalysts of C-terminal esters and amides of ubiquitin. We have found that Nedd8-AMC is a sensitive substrate for UCH-L3 (Fig. 2 and Table II), an enzyme previously shown to hydrolyze Nedd8 derivatives (18) and is generally useful as a substrate for deneddylases. This is consistent with the observations that the C-terminal amidomethylcoumarin of ubiquitin (Ub-AMC) is a sensitive and useful substrate for several deubiquitinating enzymes. It is also a specific and sensitive substrate for DEN1 as shown in Table II.

Nedd8-VS Is a Specific Inhibitor of Deneddylases—Vinyl sulfonyl derivatives are known to be potent inhibitors of thiol proteases because of the vinyl group undergoing a Michael addition by the active-site thiol. Recent work with Ub-VS and deubiquitinating enzymes confirms this expectation (31, 32). The reaction of ubiquitin vinyl sulfone was shown to be a specific and sensitive way to modify deubiquitinating enzymes. Furthermore, the reaction is exquisitely specific. Borodovsky et al. (31) showed that when cell lysates were reacted with HA-Ub-VS only deubiquitinating enzymes (and two putative DUBs) are labeled. Thus, HA-Ub-VS has wide applicability as a reagent for proteomics studies of DUBs and for discovering new DUBs.

The current work shows that Nedd8-VS is an effective and sensitive inhibitor of deneddylases. UCH-L3 is rapidly and selectively modified leading to inhibition of the hydrolysis of Nedd8-AMC (Fig. 2) and Ub-AMC (data not shown). This inhibition is no doubt because of the fact that the substrate-binding

| Protein                        | GenBank™ protein accession No. | $\mu$   | No. peptides identified |
|-------------------------------|--------------------------------|--------|------------------------|
| $\gamma\beta$-Actin           | gi|8019861                       | 40,992 | 11                     |
| ARP2/3 protein complex subunit 34 | gi|5031599                       | 34,311 | 10                     |
| Ribosomal protein L5          | gi|206734                        | 35,379 | 7                      |
| Tropomodulin 3                | gi|8394460                       | 39,478 | 6                      |
| Tropomyosin $\alpha$4         | gi|20864610                      | 28,450 | 5                      |
| SENP8                         | gi|20890310                      | 26,644 | 5                      |
| Nedd8                         | gi|4858043                       | 8,555  | 4                      |
| G-protein $\beta$             | gi|5174447                       | 35,055 | 4                      |
| Ribosomal protein S3a         | gi|7441114                       | 29,940 | 4                      |
| Nascent polypeptide-associated complex $\alpha$ | gi|20858455 | 24,582 | 4                      |
site is occupied with the reagent and the active-site thiol has been inactivated by reaction with the vinyl sulfone. Similarly, DEN1 is rapidly and quantitatively modified by Nedd8-VS (Fig. 6).

Detection and Identification of Deneddylases—In analogy to the studies with HA-Ub-VS (31), the introduction of the HA epitope tag to Nedd8-VS would open the way to identification and purification of deneddylases. However, we found that HA-tagged Nedd8 is insoluble so we turned to the FLAG epitope tag to Nedd8-VS would open the way to identification of UCH family DUBs.

The 40-kDa band shown in Fig. 3 was identified by LC MS/MS as UCH-L3. This result is consistent with previous observations of UCH-L3 reactivity (18) and our studies with Nedd8-AMC (Table I). We have confirmed that both recombinant UCH-L3 and UCH-L1 are efficiently labeled by FLAG-Nedd8-AMC (data not shown). The ability of UCH-L3 to bind and hydrolyze Nedd8 is because of the fact that the face of ubiquitin that is bound to UCH-L3 is highly conserved in Nedd8 (42). The corresponding surfaces on SUMO-1 and ISG15 are significantly divergent explaining why these Ubl proteins are not substrates for UCH family DUBs.

Identification of the 35-kDa DEN1 Adduct—Prelabeling of the extracts with Ub-VS, followed by labeling with FLAG-Nedd8-AMC (Fig. 3) resulted in a markedly diminished labeling of UCH-L3. This is to be expected as UCH-L3 exhibits significant affinity for ubiquitin. In contrast, the 35-kDa adduct was still observed when FLAG-Nedd8-AMC was subsequently added. Thus, the protein giving rise to the 35-kDa adduct is selective for Nedd8 and reacts only minimally with Ub-VS. We called this protein DEN1. Mass spectroscopic analysis of the affinity purified 35-kDa DEN1 adduct yielded four Nedd8 peptides and five peptides from a previously uncharacterized 221-amino acid protein annotated as SENP8. This identification was confirmed with affinity purified antibodies to the 212-amino acid human SENP8. Lysates of mouse L-M(TK7) fibroblasts contain only one anti-SENP8 immunoreactive band with an apparent molecular mass of 28 kDa (predicted is 25 kDa). This protein does not react with Ub-VS under conditions where UCH-L3 is completely derivatized (Fig. 5). It is, however, quantitatively labeled by FLAG-Nedd8-AMC to form the adduct at 35 kDa. These experiments unambiguously show that DEN1 is SENP8.

DEN1 is a member of the ULP family of DUBs. ULP family members possess a core catalytic domain of ~220 amino acid, usually at the C terminus (Fig. 7). Most members of this family contain N-terminal extensions that are thought to be involved in determining the substrate specificity or localization of these enzymes (22, 43, 44). Yeast contains two ULPs and both hydrolyze SUMO-1 and SUMO-2/3 derivatives, although with different apparent specificity and nuclear localization (23, 45).

### Table II

|       | DEN1       |          | UCH-L3       |          | Pro-Nedd8 |
|-------|------------|----------|--------------|----------|-----------|
|       | Nedd8-AMC  | Ub-AMC   | Nedd8-AMC    | Ub-AMC   | Pro-Nedd8 |
|       |            |          |              |          |           |
| $K_m$ | 51 nM      | >5,000 nM| 44 nM        | >5,000 nM| >5,000 nM |
| $K_m$ | 185 nM     | >5,000 nM| 350 nM       | ND       | ND        |
| $k_{cat}/K_m$ | 1.3 x 10^6 M⁻¹ s⁻¹ | 2.2 x 10⁸ M⁻¹ s⁻¹ | 1.4 x 10⁶ M⁻¹ s⁻¹ | 1 x 10⁷ M⁻¹ s⁻¹ | 1 x 10⁶ M⁻¹ s⁻¹ |

There are seven members of this gene family in humans, although little is known about the specific roles of these enzymes (46). Family members contain eight blocks of highly conserved sequence in the catalytic domain (Fig. 7). These motifs (determined independently by sequence analysis) correspond perfectly to the major secondary structural units (44). The crystal structure of the core catalytic domain yeast ULP1 in a complex with SMT3 (the yeast SUMO homolog) reveals that this protein binds primarily to the C-terminal 30 residues of the SMT3 Ubl domain. The sequence of this region is markedly different from that of ubiquitin, Nedd8, and ISG15 and this is thought to be the reason why all ULPs characterized to date show specificity for SUMO and not the other ubiquitin-like proteins. Thus, it is surprising that SENP8 would have significant activity toward Nedd8. For this reason we more carefully characterized the kinetics of catalysis by DEN1.

Substrate Specificity of DEN1—DEN1 catalyzes the hydrolysis of Nedd8-AMC and Ub-AMC, but not SUMO1-AMC (Table I). DEN1 exhibits a $K_m$ of about 50 nM for Nedd8-AMC suggesting that Nedd8 derivatives could be physiological substrates. The value of $k_{cat}/K_m$ suggests that binding is rate-limiting for this substrate with a $k_{cat}$ of 7 s⁻¹. The hydrolysis of Ub-AMC by DEN1 exhibits a $K_m$ of greater than 5 μM. Because we could not saturate the enzyme with Ub-AMC we cannot determine the $k_{cat}$ value, but it can be estimated to be greater than 0.02 s⁻¹. The actual binding constants for Nedd8 and ubiquitin were determined by competitive inhibition to be 185 nM and >5 μM, respectively (Table II). SUMO-1 is not a substrate and DEN1 does not bind SUMO-1 (47).

Conversely, UCH-L3 prefers ubiquitin substrates by a similar magnitude over Nedd8 substrates (Table II). Ub-AMC hydrolysis by UCH-L3 exhibits a $K_m$ of 44 nM and a $k_{cat}$ of 6.5 s⁻¹, essentially identical to those for DEN1-catalyzed hydrolysis of Nedd8-AMC. The binding constant for ubiquitin binding to UCH-L3 is 350 nM, consistent with the diffusion-limited binding of substrate. UCH-L3 hydrolyzes Nedd8-AMC and pro-Nedd8 with $k_{cat}$ values of greater than 0.5 and 5 s⁻¹, respectively, although there is no evidence of satureable binding at concentrations up to 5 μM. Thus, UCH-L3 is capable of hydrolyzing Nedd8 derivatives with good catalytic efficiency, although there is little affinity for such substrates. It seems likely that UCH enzymes would have to have the Nedd8 derivatives delivered by a carrier or adapter to efficiently hydrolyze such substrates.

How is substrate specificity achieved? DEN1 is a ULP family member and, where tested, the others appear to hydrolyze SUMO derivatives. It is, however, the most divergent ULP (Fig. 7A). In the catalytic core, DEN1 has more differences than similarities. These differences extend into the ULP/Ubl interface and at least seven residues that are predicted to contact the Ubl domain are substantially different from the consensus ULP sequences (Fig. 7). These include Asp-10 (G/K/R in other ULPs), Ser-11 (H/I/L), Gly-33 (N/D), Val-51 (N/S), Ghu-57 (K/Q/R), Pro-77 (K/N/H), and Ala-98 (H/N). A comparison of other DEN1 sequences shows that many of these changes are con-

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Nedd8-VS is attested to by the fact that only two proteins are tagged. The specificity of labeling of deneddylases by FLAG-site is occupied with the reagent and the active-site thiol has been inactivated by reaction with the vinyl sulfone. Similarly, DEN1 is rapidly and quantitatively modified by Nedd8-VS (Fig. 6).

Hydrolysis of Ub1-AMC substrates was determined spectrofluorometrically (36) in a final volume of 120 μL. The assays contained the indicated substrates, 50 mM Tris HCl, pH 7.6, 0.1 g/liter ovalbumin, and enzyme at a concentration of 15 pm to 50 nm as needed. The hydrolysis of pro-Nedd8 was monitored by high performance liquid chromatography (34).
served in this subfamily (47). What is striking about these changes is that most result in charge reversal or loss of H-bonding potential. Because these changes would obviate the ionic interactions that have been shown to be important for SMT3 binding to ULP1, it is easy to see why this ULP has little affinity for SUMO derivatives. Conversely, these changes would also generate a much more hydrophobic interface and such changes would be necessary to interact with the more hydrophobic C terminus of Nedd8. A more detailed analysis will require sophisticated molecular modeling and further structural studies.

**Activities and Substrates of DUBs**—Ubiquitin derivatives can be hydrolyzed by at least three DUB families (UCH, UBP/USP, and JAMM isopeptidase). Previous studies have shown that Nedd8 peptides and fusion proteins can be cleaved by UCH-L3 (18), USP21 (21), and the signalosome (26), members of the same three families. Our finding that DEN1 is a ULP adds a fourth family to those whose members can act as deneddylases. In other words, the primary sequence of the DUB places it in an evolutionarily defined family, but says little about which Ubl is the substrate (Fig. 8). To emphasize this point we have used the term deneddylase in an attempt to accurately describe the activity, as well as to avoid inferring familial relationships from enzymatic activity.

Which of the deneddylases is physiologically relevant? Most studies of DUB activities have not quantitated the specificity or selectivity of catalysis. This makes it difficult to know if they are efficient and selective catalysts. The studies reported here are the first quantitative estimates of the catalytic constants for deneddylases. Despite this detailed analysis, we do not know the physiological substrate for DEN1. This enzyme could serve a function in the salvage pathway, reclaiming Nedd8 that had been adventitiously trapped by cellular amines and thiols. Similarly, it could be involved in processing the pro-protein to yield mature Nedd8. Rub1, the yeast homolog of Nedd8, is processed by YUH1. However, deletion of both mouse homologs (UCH-L1 and UCH-L3) leads to only subtle developmental problems suggesting that enzymes other than the UCHs may play a role in this critical processing event. Finally, it is possi-
nable that DEN1 is involved in deneddylating cullins, modifiers of ubiquitin-ligase function and the only known substrate for neddylation. We have found that DEN1 is capable of efficiently removing Ned8 from hyper-neddylated cullin although not from mono-neddylated cullin (47). Thus, DEN1 may play a role in preventing accumulation of hyper-neddylated cullin and maintaining the level of mono-neddylated cullin that is thought to increase the efficiency of ubiquitination by SCF ubiquitin ligases.

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Identification and Characterization of DEN1, a Deneddylase of the ULP Family
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