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Crystal Structure of the Human Ubiquitin-like Protein NEDD8 and Interactions with Ubiquitin Pathway Enzymes*

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The NEDD8/Rub1 class of ubiquitin-like proteins has been implicated in progression of the cell cycle from G_1 into S phase. These molecules undergo a metabolism that parallels that of ubiquitin and involves specific interactions with many different proteins. We report here the crystal structure of recombinant human NEDD8 refined at 1.6-Å resolution to an *R* factor of 21.9%. As expected from the high sequence similarity (57% identical), the NEDD8 structure closely resembles that reported previously for ubiquitin. We also show that recombinant human NEDD8 protein is activated, albeit inefficiently, by the ubiquitin-activating (E1) enzyme and that NEDD8 can be transferred from E1 to the ubiquitin conjugating enzyme E2–25K. E2–25K adds NEDD8 to a polyubiquitin chain with an efficiency similar to that of ubiquitin. A chimeric tetramer composed of three ubiquitins and one histidine-tagged NEDD8 binds to the 26 S proteasome with an affinity similar to that of tetraubiquitin. Seven residues that differ from the corresponding residues in ubiquitin, but are conserved between NEDD8 orthologs, are candidates for mediating interactions with NEDD8-specific partners. One such residue, Ala-72 (Arg in ubiquitin), is shown to perform a key role in selecting against reaction with the ubiquitin E1 enzyme, thereby acting to prevent the inappropriate diversion of NEDD8 into ubiquitin-specific pathways.

Ubiquitin (Ub)[1] is a small intracellular protein of 76 amino acid residues that is found both as a monomer and covalently conjugated to other protein molecules. Conjugation results from a covalent isopeptide linkage between the C terminus of Ub and a lysine side chain(s) of the target protein. Conjugation often involves the attachment of a polyubiquitin (polyUb) chain, in which a series of Ub molecules are linked one to another through isopeptide bonds between the C terminus of one Ub and a lysine residue of the adjacent Ub (Refs. 1 and 2 and reviewed in Ref. 3). PolyUb chains linked through Lys-48 play a well defined role as a signal that targets substrate proteins to the 26 S proteasome for degradation (2, 4). In its capacity as a degradation signal Ub plays a key role both in housekeeping functions and in tightly regulated processes such as progression of the cell cycle. In the latter case, Ub and 26 S proteasome-mediated degradation accomplishes the synchronized removal of various activators and inhibitors of cyclin-dependent kinases (5). Other functions of ubiquitination, distinct from that of targeting to the 26 S proteasome, have also been identified (6, 7).

The very high conservation of the Ub amino acid sequence (only three residue substitutions between human and yeast) is presumably a function of the specific interactions that Ub makes with many other proteins. The C terminus of monomeric Ub is activated by the ubiquitin-activating (or E1) enzyme, which in turn passes the Ub to a ubiquitin conjugating (or E2) enzyme. Inspection of the *Saccharomyces cerevisiae* genome sequence indicates the presence of 13 different E2 enzymes, each of which is thought to be associated with a distinct set of substrates (8). It has recently been shown that two of these E2 enzymes mediate the conjugation of Ub-like proteins, rather than the conjugation of Ub (9, 10). A further level of ubiquitination specificity is provided by the numerous ubiquitin ligases (E3 enzymes), some of which are known to make direct contact with Ub (11). In the context of a polyUb chain, Ub also binds to one or more components of the 26 S proteasome regulatory complex. Finally, Ub also makes critical interactions with deubiquitinating enzymes, 17 of which have been identified in the genome of *S. cerevisiae* (12). Thus, the functions of Ub result from a series of highly specific macromolecular interactions. Taken together, these interactions are likely to involve most of the protein surface.

Several ubiquitin-like proteins (Ubls) have been identified that share sequence similarity with Ub. One such protein, known as NEDD8 in mammals (13, 14) and Rub1 in yeast (8), also identified in plants (15), shares ~60% sequence identity with Ub (16). Invariant residues in the Ub and NEDD8 sequences include most of those near the C terminus and most of the lysine residues, including Lys-48 which is critical for Ub's degradative signaling function (above). NEDD8 undergoes a metabolism which parallels that of Ub, including activation by a distinct E1-like enzyme (10, 17) and conjugation mediated by a dedicated E2 enzyme (10). NEDD8 and its orthologs also form conjugates with intracellular proteins, a process that, as for Ub, requires the C-terminal Gly-76 residue of the mature processed protein (10, 14, 18). One of the best characterized Ubls is the mammalian protein SUMO-1 (19, 20) and its yeast ortholog Smt3 (21). Like NEDD8, SUMO-1/Smt3 undergoes a metabolism that parallels that of Ub, with distinct E1-like (22) and E2 enzymes (9). SUMO-1 also performs an intracellular targeting function, but this function involves neither the 26 S proteasome nor degradation. At least in some cases, SUMO-1

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The atomic coordinates and structure factors (codes 1ndd and rlnddsf) have been deposited in the Protein Data Bank, Brookhaven National Laboratory, Upton, NY.

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[1] The abbreviations used are: Ub, ubiquitin; DHFR, dihydrofolate reductase; DUBs, deubiquitinating enzymes; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin-protein ligase; Ub_n, Lys-48-linked polyubiquitin chain composed of *n* ubiquitins; polyUb, polyubiquitin; Ubl, ubiquitin-like protein; His_{10}-NEDD8, recombinant human NEDD8 that contains an N-terminal extension of 10 histidine residues.

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conjugation is a signal for targeting to the nuclear pore complex (19, 20) or for specific locations within the nucleus (23). Despite the presence of only 18% sequence identity, the recently reported three-dimensional structure of SUMO-1 closely resembles that of Ub (24). Another Ubl, ISG15(UCRP), also serves a targeting role by localizing conjugated proteins to intermediate filaments (25).[2]

A possible function for NEDD8 in the regulation of cell cycle progression is suggested by the observation that the *S. cerevisiae* ortholog, Rub1, is found covalently attached to the cullin protein Cdc53 (10, 18), which is a component of the SCF^{Cdc4} ubiquitin-ligase complex. The activity of this E3 is critical for progression from G_1 to S phase (26, 27). SCF^{Cdc4}, which in addition to Cdc53 contains the Skp1 and Cdc4 proteins and the E2 enzyme Cdc34, functions by conjugating Ub to the cyclin-dependent kinase inhibitor Sic1, thereby targeting Sic1 for degradation. Yeast cells are apparently healthy following deletion of the genes for Rub1, the Rub1-specific E1-like enzyme, or the Rub1-specific E2 enzyme. Nevertheless, a role for Rub1 in SCF^{Cdc4} function is indicated by the synthetic enhancement of mutations in the *CDC34*, *CDC4*, *CDC53*, and *SKP1* genes by deletion of the *RUB1* or *ENR2* genes (*ENR2* encodes a component of the heterodimeric Rub1-specific activating enzyme). Overproduction of Cdc34 or Cdc53 also sensitizes cells to loss of the Rub1 modification. Furthermore, a C-terminal truncation of Cdc53 renders this protein resistant to Rub1 modification and at the same time makes the cells sensitive to mutation of CDC34. Cullins are also conjugated to NEDD8 in higher organisms.[3] Further evidence for a NEDD8 function in cell cycle progression is provided by a hamster cell line carrying a temperature-sensitive allele of a gene nearly identical to that encoding one of the subunits of the human NEDD8-activating enzyme. At nonpermissive temperatures these cells traverse multiple S phases without intervening mitoses (cited in Ref. 18).

In an effort to understand the biochemical and biological functions of NEDD8, we have determined the crystal structure of recombinant human NEDD8. Biochemical analysis demonstrates that Ala-72 of NEDD8 (Arg-72 in Ub), performs a key role in preventing the interaction of NEDD8 with the ubiquitin E1 enzyme. In the context of a chimeric polyUb molecule, NEDD8 is competent to interact with the 26 S proteasome. The distribution of conserved and divergent residues on the surface of the NEDD8 structure provides a framework for considering the interactions of NEDD8 with other proteins.

EXPERIMENTAL PROCEDURES

NEDD8 Plasmids—A human NEDD8-encoding gene comprising 76 amino acids was generated by PCR amplification using pcDNA/hNEDD8 (14) as template. The 5′ primer (5′-TGGAAGACATATGCTAATTAAAGTGAAG-3′) and the 3′ primer (5′-CTGGATCCTCATCCTCCTCTCAGAGCCA-3′) harbored sites for *Nde*I and *Bam*HI, respectively. The PCR product was digested with these two enzymes and ligated into pET3a (to produce pET3a-NEDD8). The sequence of the insert was verified by automated sequencing (Hopkins Core Facility). The insert was excised and ligated into pET16b to allow for the expression of a His_{10}-tagged version of NEDD8. A cDNA encoding NEDD8-A72R was created by PCR amplification with the 5′ primer described above and a 3′ primer specifying the mutation (5′-CTGGATCCTCATCCTCCTCTCAGACGCAACACCAGGTG-3′). The PCR product was cloned into pET3a as described above. The presence of the A72R mutation was verified by automated DNA sequence analysis.

Expression, Purification, and Refolding of NEDD8 Proteins—The plasmid pET3a-NEDD8 was expressed in *Escherichia coli* strain BL21(DE3)pLysS at 37 °C as described previously (28). The cell pellet from a 2-liter culture was suspended in 20 ml of lysis buffer (28); cell lysis and digestion of DNA were carried out as before (28). The crude lysate was centrifuged at 15,000 × *g* for 20 min. The pellet was resuspended in 10 ml of buffer containing 50 mM Tris-HCl (24% base, pH 7.6), 1 mM EDTA, 20% v/v sucrose, and 1% Triton X-100. NEDD8 inclusion bodies were pelleted by centrifugation at 15,000 × *g* for 20 min; this wash step was repeated twice. The purified inclusion bodies were suspended in buffer containing 50 mM Tris-HCl (24% base), 2 mM EDTA, and 8 M urea; the suspension was held at room temperature for 30 min, during which time it became clear. The solution was then dialyzed extensively at 5 °C against buffer containing 50 mM Tris-HCl (24% base), 2 mM EDTA, and 1 mM dithiothreitol; any precipitate that formed was removed by centrifugation. The protein solution was passed successively through 10-ml columns of 1) Q-Sepharose and 2) SP-Sepharose (both from Amersham Pharmacia Biotech) which had been pre-equilibrated with dialysis buffer. The final flow-through fraction was concentrated (Millipore Ultra-free 4) to yield a NEDD8 concentration of ~20 mg/ml. Aliquots (1 ml) of the concentrated protein were run on a 1 × 50-cm Sephacryl-200 column (Amersham Pharmacia Biotech) pre-equilibrated with dialysis buffer. Fractions (1 ml) were collected, and aliquots were analyzed by SDS-PAGE to identify the peak fractions. Usually about 30 mg of purified NEDD8 was recovered from 1 liter of cell suspension; the purity of the protein was >98% as evaluated by SDS-PAGE and Coomassie staining. Sometimes a fraction of the NEDD8 protein precipitated during repeated cycles of freezing and thawing.

NEDD8-A72R was expressed and purified as described above, with the following differences. 1) The refolded mutant protein bound weakly to SP-Sepharose. Therefore the loaded column was washed with dialysis buffer containing 0.1 M NaCl prior to elution with buffer containing 0.15 M NaCl. 2) The gel filtration step was omitted. The purified mutant protein was >90% pure by SDS-PAGE and Coomassie staining. About 5 mg of purified NEDD8-A72R was obtained from 1 liter of cell suspension.

His_{10}-NEDD8 was expressed as described above. The cell pellet from a 400-ml culture was resuspended in 8 ml of buffer containing 50 mM Tris-HCl (24% base) and 8 M urea. The suspension was held for 1 h at 60 °C, during which time the cells lysed. The lysate was passed through a 2.5-ml Ni^{2+}-nitrilotriacetic acid column (Novagen). The loaded column was washed extensively with the same buffer, and His_{10}-NEDD8 was eluted with 3 volumes of the same buffer containing 0.2 M imidazole. The protein was dialyzed and concentrated (above).

E1 and E2s—Ubiquitin-activating enzyme (E1) was purified from rabbit reticulocytes (29). The following E2s were purified from bovine erythrocytes or rabbit reticulocytes: E2–14K (29), E2–17K (30), E2–20K (29), and E2–35K (29). Purified recombinant human Ubc13 homolog was a gift of R. Hofmann (Johns Hopkins); purified recombinant human UbcH5B (31) was a gift of J. You (Johns Hopkins). C170S-E2–25K was expressed and purified as described (28, 32).

E2~Ubiquitin and E2~NEDD8 Thiol Ester Formation—Bovine Ub (Sigma) and recombinant NEDD8 (above) were radioiodinated to ~9000 and ~6000 cpm/pmol, respectively (33). Thiol ester assays were carried out at pH 7.3 and 37 °C as described previously (28), using 4 μM labeled protein and ~3 μM E2. The concentration of E1 was 0.1 μM (Ub assays) or 0.5 μM (NEDD8 assays). Assays (8 μl) were quenched after 3.5 min. (Ub) or 25 min (NEDD8) with an equal volume of sample buffer lacking β-mercaptoethanol. The E2 thiol esters were detected by electrophoresis and autoradiography and quantitated by band excision and γ-counting. The formation of a labile E2~NEDD8 adduct was observed with each E2, but the appearance of the NEDD8 thiol ester (at 0.5 μM E1) was much slower than the appearance of the corresponding Ub thiol ester (at 0.1 μM E1). Ubc13 gave a higher yield of NEDD8 thiol ester than did the other E2s assayed.

Pulse-Chase Assays with E2–25K—Pulse incubations (pH 7.3 and 37 °C) contained ~4 μM C170S-E2–25K and 4 μM ^{125}I-Ub or ^{125}I-NEDD8. The conditions of the 10-min pulse, and the concentration of E1, were the same as in thiol ester assays (28). At the end of the pulse, a 2.5-μl aliquot was removed to monitor thiol ester formation. The chase was then initiated by adding a mixture providing 1 mg/ml of unlabeled Ub or NEDD8 and 10 mM EDTA. Aliquots were quenched at increasing times in sample buffer without mercaptoethanol and analyzed by SDS-PAGE and autoradiography. Pseudo-first order rate constants for the disappearance of the E2 thiol ester were obtained from semi-log plots of thiol ester radioactivity *versus* time.

E1 Competition Assay—Incubations were carried out under the same conditions as for E2 thiol ester formation, except that E2 was omitted and the concentration of E1 was 0.15 μM. ^{125}I-Ub was 1.3 μM; the concentration of unlabeled NEDD8 or Ub was varied (see "Results"). The reaction was initiated by adding E1 and quenched after 3 min with sample buffer lacking mercaptoethanol. The E1~^{125}I-Ub thiol ester was

[2] A. L. Haas, personal communication.
[3] E. T. H. Yeh, personal communication.

detected following electrophoresis and autoradiography and quantitated by band excision and counting.

Synthesis of His_{10}-NEDD8-Ub_3—E2–25K was used to conjugate His_{10}-NEDD8 to Lys-48 at the distal terminus of Lys-48-linked Ub_3. The Ub_3 was purified by cation exchange from a mixture of chains assembled using E2–25K (34); it was largely des-GlyGly at its proximal terminus, which prevented self-conjugation. The incubation (200 μl, pH 7.3, and 37 °C) contained 5 μM C170S-E2–25K, 0.25 mM dithiothreitol, 0.2 μM E1, 58 μM Ub_3, and 150 μM His_{10}-NEDD8. Other conditions were as described previously (32). Incubation was for 2 h (37 °C), and 0.2 μM additional E1 was added at 40 and 80 min. The poor acceptor activity of NEDD8 ("Results") prevented the ligation of more than one His_{10}-NEDD8 to the chain. The chimeric His_{10}-NEDD8-Ub_3 tetramer (and free His_{10}-NEDD8) were resolved from unutilized Ub_3 and enzymes by chromatography on nickel resin. Bovine serum albumin was added to the column eluate as a carrier prior to concentration and buffer exchange. Due to the small amount of chimeric tetramer, we did not attempt to purify this species further. Based on the failure of mono-Ub to inhibit the 26S proteasome (35), we considered it unlikely that the residual mono-Nedd8 would interfere in our assays, and this proved to be the case ("Results").

Competition Assay for Chain Binding to 26 S Proteasome—The competition assay was similar to that described previously (35), except that it employed a substrate in which Lys-48-linked Ub_4 was conjugated to Lys-48 of the Ub moiety in a linear Ub-DHFR fusion protein (the DHFR fusion protein was metabolically labeled with ^{35}S-Met, in *E. coli)*. Degradation was monitored by the appearance of acid-soluble radioactivity and was linear in time and proteasome concentration. The properties of this substrate, and the assay, will be described in detail elsewhere.[4] Purified 26 S proteasome was generously provided by L. Hoffman and M. Rechsteiner (University of Utah). Ub_4 was synthesized as described (35).

Crystallization and X-ray Data Collection—Recombinant human NEDD8 was crystallized by vapor diffusion in sitting drops at 21 °C. The initial protein solution was 10 or 15 mg/ml NEDD8, 40 mM Tris, pH 7.6, 50 mM NaCl, 0.4 mM EDTA, and 0.2 mM dithiothreitol. The reservoir solution was 500 μl of 100 mM citric acid, pH 4.8, and 2.2 M ammonium sulfate. Drops were made by mixing 2 μl each of protein and reservoir solutions. Clusters of thin crystalline plates formed after 24 to 36 h, and small prism-shaped crystals up to 0.01 mm in the longest dimension appeared a few days later. The small prism-shaped crystals were transferred to fresh crystallization drops that had equilibrated for less than 24 h. Crystals grew from these seeds to typical dimensions of 0.1 × 0.1 × 0.1 mm.

Two closely related crystal forms with indistinguishable morphology grew in the same drop. The two crystal forms belong to space groups P1 and $P2_1$, and they both contain 4 molecules in the asymmetric unit. The P1 crystals diffracted to approximately 2.10-Å resolution and had unit cell dimensions a = 34.1 Å, b = 45.3 Å, c = 48.4 Å, α = 83.8°, β = 73.2°, γ = 79.5°. The $P2_1$ crystals diffracted to 1.60-Å resolution and had unit cell dimensions a = 45.8 Å, b = 65.0 Å, c = 48.6 Å, β = 96.6°. The P1 and $P2_1$ crystals are closely related to each other and have very similar Matthews' V_m coefficients (36) of 2.06 and 2.10 Å^3/Da, which correspond to solvent contents of 39.7 and 41.0%, respectively.

All x-ray diffraction data were collected at −170 °C using a copper rotating anode source and an RAXIS-IV image plate area detector. Prior to data collection the crystals were immersed for 2 ($P2_1$) or 30 (P1) min in a solution containing 100 mM citric acid, pH 4.8, 2.6 M ammonium sulfate, and 15% glycerol. The crystals were suspended in a small rayon loop attached to a metal pin and cryo-cooled by plunging into liquid nitrogen. The crystals were transferred to the data collection instrument, and complete data sets were collected from each of the crystals. Data were collected as 0.6 to 1.0° rotation images with typical exposure times of 10–60 min. Data were indexed, integrated, and scaled with DENZO and SCALEPACK (37). Data collected from crystal form P1 extend to 2.10-Å resolution with an R_{sym} of 10.6%. The data from crystal form $P2_1$ extend to 1.60-Å resolution with an R_{sym} of 6.7%. See Table I for data processing statistics.

Structure Determination and Refinement—Most crystallographic computations used programs from the Collaborative Computing Project 4 suite (38). The scaled diffraction intensities were converted to structure-factor amplitudes using the program TRUNCATE (39). Structure determination was by Molecular Replacement using the program AMoRe (40). A single molecule of Ub (41) (PDB entry 1ubq) (modified by deleting the C-terminal 6 residues) was used as the search probe. This gave a clear solution against the P1 data; fitting of all four molecules in the asymmetric unit gave a correlation coefficient of 0.327 and an R factor of 48.5% against data in the resolution range 8.0–3.5 Å.

Refinement was performed using the program XPLOR (42). Preliminary refinement against the P1 data of a NEDD8 model consisting of residues 12 through 70 resulted in a crystallographic R factor of 32.5% and free R factor of 44.5%. This partially refined model was used as a probe to determine the structure of the $P2_1$ crystal form by Molecular Replacement (40) (correlation coefficient = 0.596, R factor = 40.6%, for data in the resolution range 6.0–3.5 Å).

Both the P1 and $P2_1$ crystal form structures have been refined by torsion angle dynamics, simulated annealing, positional, and B-factor refinement. Rounds of automatic refinement calculations were interspersed with manual rebuilding into simulated-annealing $2F_o - F_c$ and $F_o - F_c$ omit maps using the program O (43). For both crystal forms, refinement gave models that have acceptable agreement with both the diffraction data and with expected stereochemistry. The refined model for the P1 crystal form has an R factor of 21.9% (free R factor 33.0%), and the refined model for the $P2_1$ crystal form has an R factor of 21.9% (free R factor 29.4%). All data were used in these calculations without application of a cutoff based on the estimated standard deviation. Almost all residues are well defined in electron density maps. The following residues are partially disordered: crystal form P1, molecules 1–3, residues 74–76; molecule 4, residue 76: crystal form $P2_1$, molecule 1, residues 75 and 76, molecule 3, residue 76, molecule 4, residues 74–76. A bulk solvent correction was applied in XPLOR for the final three rounds of refinement and map calculation. Non-crystallographic symmetry restraints were not applied during the refinement. Refinement statistics are given in Table II.

TABLE I
Data collection statistics

Data were collected on a rotating anode x-ray source using an RAXIS-IV image plate detector. Values in parentheses refer to the highest resolution shell.

Crystal form	P1	$P2_1$
No. of reflections observed	76,227	232,354
No. of reflections unique	15,223	36,579
d_{min} (Å)	2.10	1.60
High resolution shell	2.10–2.14	1.60–1.63
Completeness (%)	96.0 (93.8)	97.8 (93.7)
R_{sym} (%)[a]	10.6 (36.8)	6.7 (45.2)
Average $I/\sigma(I)$	9 (2.5)	>20 (1.9)
Mosaicity (°)	0.752	0.449

[a] $R_{sym} = 100 * \Sigma|I - \langle I\rangle|/\Sigma I$.

RESULTS AND DISCUSSION

Interactions of NEDD8 with Ubiquitin Pathway Enzymes—Among the Ubl's, NEDD8 is by far the most similar to Ub (57% identical; 76% similar). In view of this high similarity, we addressed whether NEDD8 could interact productively with enzymes of the Ub conjugation cascade. In pilot studies we found that ^{125}I-NEDD8 formed a thiol ester with highly purified ubiquitin-activating enzyme (E1), as indicated by MgATP-dependent formation of a radioactive band which migrated at ~120-kDa in SDS-PAGE and was labile to treatment with mercaptoethanol (data not shown, but see Fig. 2*B* below). Despite the qualitative competence of NEDD8 in the E1 reaction, E1, and a number of other ubiquitin enzymes, interacted poorly with NEDD8 (below; see also "Experimental Procedures"). These studies predated the recent discovery of an activating enzyme and a conjugating enzyme specific for NEDD8 (10, 18). The existence of these enzymes provides an explanation for the poor utilization of NEDD8 by the ubiquitin conjugation system. However, the results of our studies of artificial NEDD8 activation provided insights into the structural basis for discrimination between the highly conserved Ub and NEDD8 molecules.

The interaction of NEDD8 with E1 was quantitatively evaluated by competition against a subsaturating concentration of ^{125}I-Ub in end point assays of E1~Ub thiol ester formation (30). Competition by NEDD8 was weak; the line shown in Fig. 1 assumes half-maximal inhibition at K_{app} = 182 μM (*open*

[4] J. Piotrowski, L. Hoffman, M. Rechsteiner, and C. M. Pickart, manuscript in preparation.

TABLE II
Refinement statistics

	P1	$P2_1$
Resolution range (Å)	20.0–2.10	20.0–1.60
Number of protein atoms[a]	2324	2352
Number of solvent atoms[a]	107	193
R factor (%)[b]	21.9	21.9
Free R factor (%)[c]	33.0	29.8
Bond lengths (Å)[d]	0.006	0.006
Bond angles (°)[d]	1.623	1.250
⟨B⟩ (Å^2) main chain	23.01	18.99
⟨B⟩ (Å^2) side chains	26.44	24.98
⟨B⟩ (Å^2) water molecules	37.00	36.13
⟨B⟩ (Å^2) chloride/sulfate ions[e]		18.04/48.25
No. of φ/ψ angles (%):[d]		
Most favored	94.0	96.9
Additional	6.0	3.1

[a] Non-hydrogen atoms only.
[b] R factor = $100 * \Sigma(||F_p(\text{obs})| - |F_p(\text{calc})||)/\Sigma|F_p(\text{obs})|$. All data were used in the resolution range indicated, without application of a cut based upon the estimated standard deviation.
[c] Free R factor = R factor for a selected subset of ~1000 reflections that were not included in prior refinement calculations.
[d] Stereochemistry was assessed with PROCHECK (61).
[e] Two chloride and four sulfate ions have been included in the $P2_1$ model.

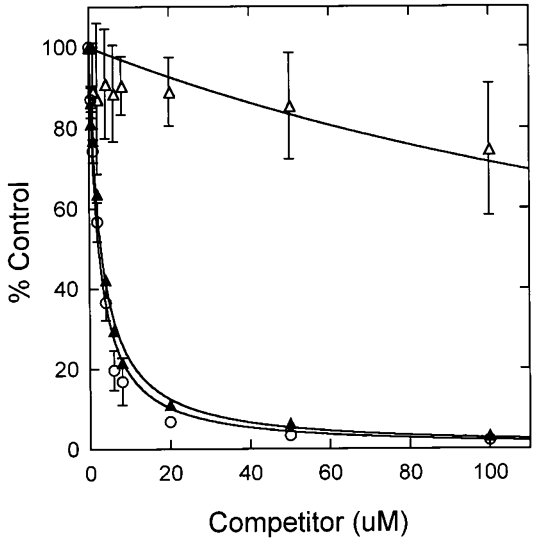

FIG. 1. **NEDD8 binding to E1.** End point assays of E1~Ub thiol ester formation were carried out as described under "Experimental Procedures." ^{125}I-Ub (1.3 μM) was mixed with the indicated concentration of unlabeled competitor prior to addition of purified E1. The concentration of labeled E1~Ub thiol ester was determined after 3 min. The data were fit assuming hyperbolic behavior using the program SigmaPlot. The lines assume half-maximal inhibition (K_{app}) at 2.0 ± 0.2 μM Ub (*open circles*), 2.8 ± 0.2 μM NEDD8-A72R (*filled triangles*), or 182 ± 47 μM wild-type NEDD8 (*open triangles*).

triangles, Fig. 1). As expected, unlabeled Ub competed efficiently (*circles*, Fig. 1). Taking into consideration the presence of labeled Ub at a concentration of 1.3 μM (legend, Fig. 1), the observed value of K_{app} for unlabeled wild-type Ub, 2.0 μM, is similar to the value expected based on binding of Ub to E1 with an intrinsic K_d of 0.4 μM (44). Among the residues that are divergent between NEDD8 and ubiquitin, Ala-72 appeared likely to contribute to the weak binding of NEDD8. An Arg-to-Leu mutation at residue 72 reduces the affinity of Ub for E1 by 100-fold (44); a similar factor of 90-fold is seen when the binding of NEDD8 is compared with that of Ub (above). We therefore constructed the Ala to Arg variant, NEDD8-A72R, and tested this mutant in the E1 competition assay. Remarkably, NEDD8-A72R bound to E1 almost as well as wild-type Ub (K_{app} = 2.8 μM for NEDD8-A72R *versus* 2.0 μM for Ub; *filled triangles versus circles*, Fig. 1). As expected, NEDD8-A72R formed a thiol ester with E1 (data not shown). These results identify Ala-72 of NEDD8 as a residue that prevents the interaction of NEDD8 with the Ub conjugation system. Ala-72 is also likely to be a point of discrimination by DUB enzymes (see below).

The Ub-like protein SUMO-1 does not appear to be involved in targeting to the 26 S proteasome. However, NEDD8 is much more similar to Ub than is SUMO-1. In view of this higher similarity, we sought to address NEDD8's proteolytic signaling ability. Therefore we analyzed the properties of NEDD8 in the reaction catalyzed by E2–25K. This E2 enzyme can assemble Lys-48-linked polyUb chains from free Ub (30). In steady-state assays E2–25K showed only weak activity toward NEDD8 (data not shown). However, slow NEDD8 activation by E1 (above) could have masked substantial competence of NEDD8 in the E2–25K reaction. Therefore we turned to pulse-chase assays, in which interference from upstream steps is avoided by starting with the preformed E2 thiol ester, and limiting the reaction to a single turnover.

Fig. 2*A* shows a control experiment in which ^{125}I-Ub was transferred from E2–25K to unlabeled Ub; Fig. 2*B* shows an experiment in which ^{125}I-NEDD8 was transferred to the same acceptor. Unexpectedly, NEDD8 was a better substrate for transfer than Ub, by a factor of five (Fig. 2*C*). Similar experiments showed that the transfer of either Ub or NEDD8 to NEDD8 was at least 50-fold slower than transfer of either species to Ub. Thus, discrimination by E2–25K between NEDD8 and Ub varies depending on the mechanistic role of the "ubiquitin": NEDD8 apparently has all of the determinants necessary for rapid transfer from E2–25K to a good acceptor, but NEDD8 lacks specific determinants of acceptor function.

The finding that there is no intrinsic block to the utilization of NEDD8 as the donor ubiquitin (Fig. 2) implies that any noncovalent interactions between the thiol-linked Ub and E2–25K are mediated by conserved features of the NEDD8/Ub molecules. This suggests that the selectivity of an E2 enzyme for Ub *versus* a particular Ubl is determined at the level of the upstream activating enzymes, rather than at the level of the molecule being transferred. E1's high selectivity for Ub (Fig. 1), its evident selectivity for Ub-dedicated E2s, and the probable converse selectivities of the NEDD8-activating enzyme, will combine to prevent the improper utilization of NEDD8 by the Ub conjugation pathway. These considerations emphasize the likely importance of specific activating enzymes in permitting Ubls to execute specific functions within cells. Selectivity of the activating enzyme is probably particularly important in the case of NEDD8, given NEDD8's high similarity to Ub.

Proteolytic Signaling by a Chimeric NEDD8-Polyubiquitin Chain—Targeting of ubiquitinated substrates to the 26 S proteasome is predominantly mediated by polyUb chains assembled through Lys-48 (2, 45). All of the known molecular determinants of proteolytic signaling by such chains are present in NEDD8, including Lys-48, a cluster of surface hydrophobic residues (Leu-8, Ile-44, Val-70), and most of the charged and polar residues whose interactions stabilize the specific conformation seen in the crystal structure of Ub_4 (2, 33, 46, 47). This conformation appears to be important for displaying determinants of interaction with the 26 S proteasome, including the hydrophobic cluster, on the chain surface (33). With the exception of Arg-72, all of the residues that stabilize this conformation (47) are either present in NEDD8 or substituted by residues that could mediate similar interactions.

NEDD8's properties in the E2–25K reaction were such that we could not assemble a NEDD8 homopolymer from either wild-type NEDD8 or NEDD8-A72R (the mutant protein was also a poor substrate for E2–25K as assessed by steady-state

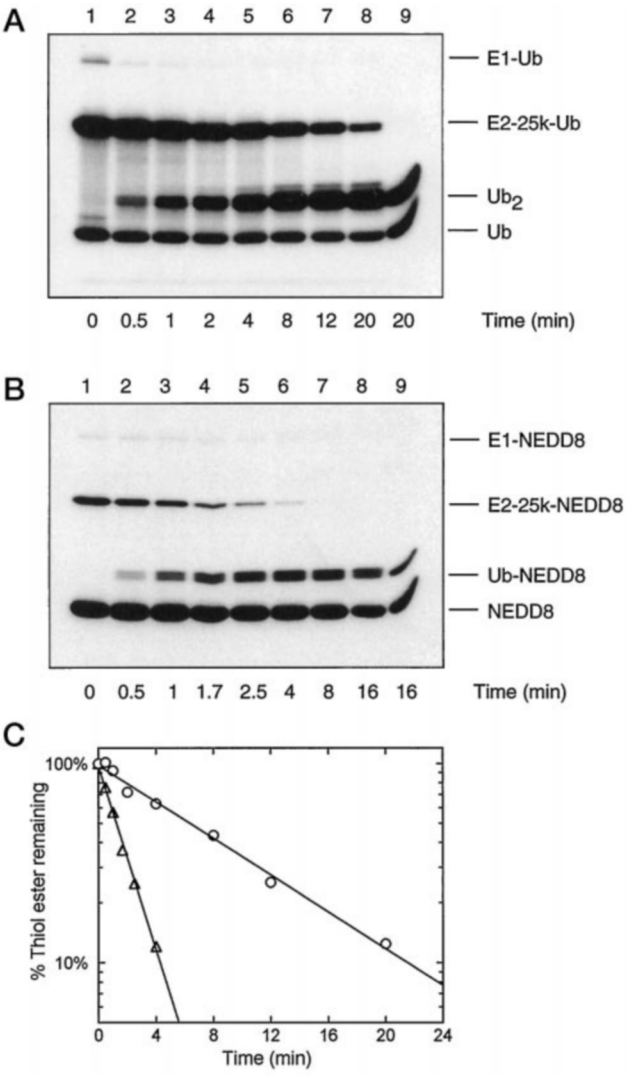

FIG. 2. **NEDD8-ubiquitin conjugation by E2–25K.** Pulse-chase assays were carried out as described under "Experimental Procedures." *A* and *B*, autoradiographs. Transfer of ^{125}I-Ub and ^{125}I-NEDD8 to Ub (1 mg/ml) is shown in *A* and *B*, respectively. In each panel, *lane 1* shows the E2 thiol ester at the end of the pulse incubation; *lanes 2* through *8* show increasing time points (see *bottom* of each panel) in the chase incubation; and *lane 9* shows an aliquot (same as in *lane 8*) in which the sample was boiled in mercaptoethanol prior to electrophoresis (other aliquots were quenched in sample buffer without mercaptoethanol). The various labeled species are identified on the right. C, analysis of data from *A* and *B*. The E2 thiol ester bands were excised and counted: *circles*, Ub transfer; *triangles*, NEDD8 transfer. The data for Ub ($t_{1/2}$ ~6 min) correspond to k_{cat}/K_m~990 M^{-1} min^{-1}, in agreement with expectation (28).

assays). However, E2–25K efficiently conjugated NEDD8 to the distal terminus of Lys-48-linked Ub_3 (see "Experimental Procedures"). To facilitate the purification of the resulting chimeric tetramer, we utilized His_{10}-NEDD8 in this reaction. In steady-state assays His_{10}-NEDD8 was utilized by E2–25K with kinetics similar to those for wild-type NEDD8 (data not shown), consistent with our finding that E1 and E2–25K do not discriminate between His_6-Ub and wild-type Ub.[5] Polyubiquitin chains assembled from His_6-Ub are efficiently recognized by the 26 S proteasome.[5] To evaluate whether the chimeric His_{10}-NEDD8-Ub_3 tetramer would bind to the 26 S proteasome, we tested this chain's ability to inhibit the degradation of a purified, radiolabeled Ub conjugate (Ref. 35; see "Experimental Procedures").

At low concentration the chimeric tetramer (0.5 μM) inhibited substrate degradation by about 60% (Exp. 3, Table III). This inhibition was somewhat stronger than that caused by the same concentration of authentic Ub_4 (Exp. 1). At this low concentration, Ub_3 does not detectably inhibit (Exp. 2). The chimeric tetramer preparation contained a 10-fold molar excess of His_{10}-NEDD8 monomer (see "Experimental Procedures"); as expected, this species made a minimal contribution to the observed inhibition (Exp. 4, Table III). Under the conditions of this assay, authentic Ub_4 is a competitive inhibitor which binds in the expected saturating fashion.[4] Assuming similar properties of the chimeric tetramer, and correcting for the low contribution of the contaminating His_{10}-NEDD8 monomer, the chimeric tetramer binds about three times more tightly than authentic Ub_4 ($K_{0.5}$ values of 0.5 and 1.6 μM, respectively; Table III). These results indicate that the distal His_{10}-NEDD8 in the chimeric tetramer functions as well as, or better than, Ub at the same position (Exp. 1 *versus* Exp. 3). This result suggests that NEDD8 has all of the determinants necessary for targeting to the 26 S proteasome in the context of a Lys-48-linked chain.

NEDD8 Crystal Structure Determination—The crystal structure of recombinant human NEDD8 was determined by molecular replacement as described under "Experimental Procedures." Two crystal forms were obtained in space groups P1 and $P2_1$. However, these two crystal forms are very similar to each other, with the only difference being a subtle alternate packing arrangement. Therefore, only the $P2_1$ structure, which diffracts to higher resolution (1.60 Å), will be described in detail. There are 4 molecules in the asymmetric unit with a solvent content of 40%. The structure has been refined to an *R* factor of 21.9% (free *R* factor 29.8%) with good stereochemistry (see Table II). As expected, the structure is very similar to that of Ub and is comprised primarily of an α-helix packed against an antiparallel β-sheet (Fig. 3).

NEDD8 molecules pack as closely associated dimers in the crystal, with two dimers in the asymmetric unit and identical dimers seen in both P1 and $P2_1$ crystal forms. Dimer formation apparently buries a total of 1500 $Å^2$ of solvent accessible surface area. Despite the extensive, close-packed dimer interface, we consider it very unlikely that noncovalent dimerization may have physiological relevance, since analysis by equilibrium sedimentation revealed that NEDD8 is a monomer at 12 mg/ml, with no evidence of self-association (data not shown).

The four crystallographically unique NEDD8 molecules adopt very similar conformations. The root mean square deviation after least squares overlap on the C^α atoms of residues 1–72 ranges from 0.4 to 0.5 Å for pairs of NEDD8 molecules. The largest difference between noncrystallographically related NEDD8 molecules is for residue 62, which in one case shows a displacement of 1.8 Å. The main chain conformation is conserved between the noncrystallographically related NEDD8 molecules, and visual inspection shows that the conformations of side chains are essentially identical, with the only differences limited to surface groups that are likely to be disordered in solution.

Comparison of the NEDD8 Structure with Ubiquitin—As expected from the high level of sequence conservation (Fig. 4), the structure of NEDD8 closely resembles that of Ub. Alignment of five crystallographically distinct Ub structures (41, 47, 48) with each of the four NEDD8 molecules in the asymmetric unit typically gives root mean square deviations of 0.75 Å for the C^α atoms of residues 1–72. Alignment of the five crystallographically distinct Ub structures with each other over the same range gives root mean square deviations that are very

[5] G. Xia and C. M. Pickart, unpublished experiments.

TABLE III
Chimeric tetramer inhibits 26 S proteasome

Purified 26 S proteasome was assayed using Ub_4-^{35}S-UbDHFR (150 nM) as substrate and the indicated potential inhibitors (see "Experimental Procedures"). Initial rates of substrate degradation are expressed relative to the rate observed in a control incubation lacking inhibitor. Stronger inhibition by the chimeric tetramer than by authentic Ub_4 was verified in an independent experiment (data not shown).

Exp.	Inhibitor	Concentration	Inhibition	$K_{0.5}$[a]
		μM	%	μM
1	Ub_4	0.5	23.5 ± 6.1[b]	1.6
2	Ub_3	0.5	0	
3	His_{10}-NEDD8-Ub_3[c]	0.5	62.0 ± 3.0[b]	0.31 (0.50[d])
4	His_{10}-NEDD8	5.5	11.9	40.0

[a] Calculated assuming inhibitor binds in a saturating fashion (verified experimentally for Ub_4 and higher concentrations of Ub_3).[4]
[b] Average of duplicate determinations.
[c] Contains 11-fold molar excess of His_{10}-NEDD8.
[d] Value of $K_{0.5}$ corrected for contribution of mono-His_{10}-NEDD8 in chimeric tetramer (see Exp. 4).

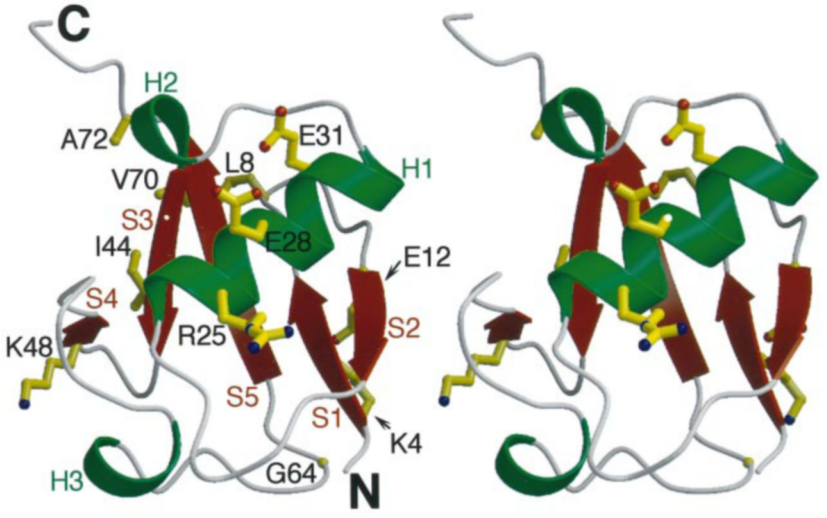

FIG. 3. **Stereoview ribbon representation of NEDD8.** N and C termini are labeled *N* and *C*. Secondary structure was defined with PROMOTIF (59). Helices are colored *green*: helix 1, residues 23–34; helix 2, 38–40; helix 3, 56–59. Helix 1 is type α, whereas helices 2 and 3 are type 3_{10}. According to the strict definitions used by PROMOTIF, helix 2 does not occur in two of the four NEDD8 molecules in the asymmetric unit, although the conformations are close to those of standard helices. β strands are colored *red*: strand 1, residues 2–6; strand 2, 12–16; strand 3, 41–45, strand 4, 48–49; strand 5, 66–71. Strand 1 has one extra residue in one of the four molecules in the asymmetric unit. Residues discussed in the text are shown explicitly.

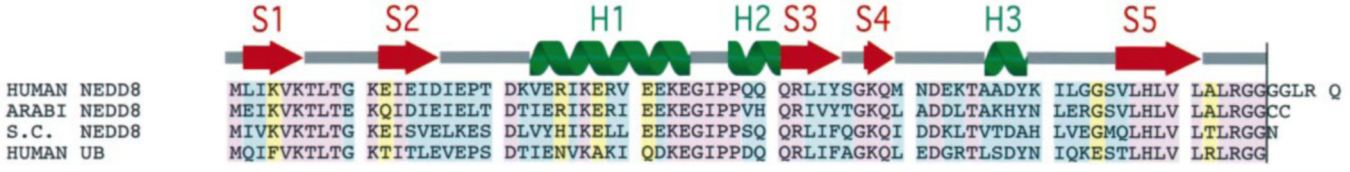

FIG. 4. **Amino acid sequence alignment.** The secondary structure of NEDD8 is shown above. Every 10th residue is followed by a space. A *vertical black line* indicates the C termini of the mature proteins. *ARABI* denotes *Arabidopsis thaliana*; *S.C.* denotes *S. cerevisiae.* Invariant residues are shown on a *magenta* background. Residues that are conserved between the NEDD8 sequences but differ from Ub (conserved/divergent residues) are shown on a *yellow* background. Divergent residues are shown on a *blue* background.

similar, typically about 0.8 Å. For most pairwise comparisons between different Ub structures, the largest deviation is seen for the β-hairpin structure of residues 8 and 9, whose C^{α} atoms differ by up to 5.0 Å. In contrast, the four NEDD8 molecules are relatively similar to each other in this region, with a maximal displacement of 1.5 Å. The Leu-8, Thr-9 β-turn of NEDD8 resembles that seen in several of the ubiquitin structures, with the closest similarity between Leu-8 C^{α} of a NEDD8 and a Ub structure being 0.8 Å, and the greatest difference being 5.4 Å. It is likely that Leu-8 and surrounding residues of NEDD8 will in fact experience a range of conformations in solution, similar to the range seen in the different Ub crystal structures.

The C-terminal residues of Ub project away from the core of the globular structure, and in the case of most Ub crystal structures, the last three residues (Arg-74, Gly-75, Gly-76) are disordered. The situation is very similar for the NEDD8 C terminus, which is also highly exposed and projects away from the body of the globular domain. One of the four NEDD8 molecules in the asymmetric unit does have ordered C-terminal residues that pack against a neighboring molecule in the crystal lattice. The last three residues of the other three molecules are not visible in electron density maps, presumably because of disorder. Therefore, as in Ub, the NEDD8 C-terminal residues will be flexible and accessible in solution.

Ubiquitin displays an asymmetric distribution of charged residues, and the structure has been described as possessing "acidic" and "basic" faces (49). Although the precise boundaries of these faces are ill defined, we estimate that the acidic face (on the same side of the structure as helix 1) possesses eight Glu/Asp and four Lys/Arg side chains, respectively, while the basic face possesses no Asp/Glu and six Lys/Arg residues, and a relatively large fraction of surface hydrophobic residues. The surface of NEDD8 displays an almost identical arrangement of acidic and basic faces (Fig. 5). This conserved electrostatic distribution persists despite the conservation of only 17 out of 30 charged residues between human NEDD8 and Ub. One notable difference in the electrostatic potential surface is the presence of a distinct positive region in the middle of the NEDD8 acidic face (*top left panel* of Fig. 5). This is due, in part, to Arg-25 of NEDD8, which is invariably Arg/His in the NEDD8 orthologs but Asn in Ub (Fig. 4).

The three most exposed hydrophobic ubiquitin residues,

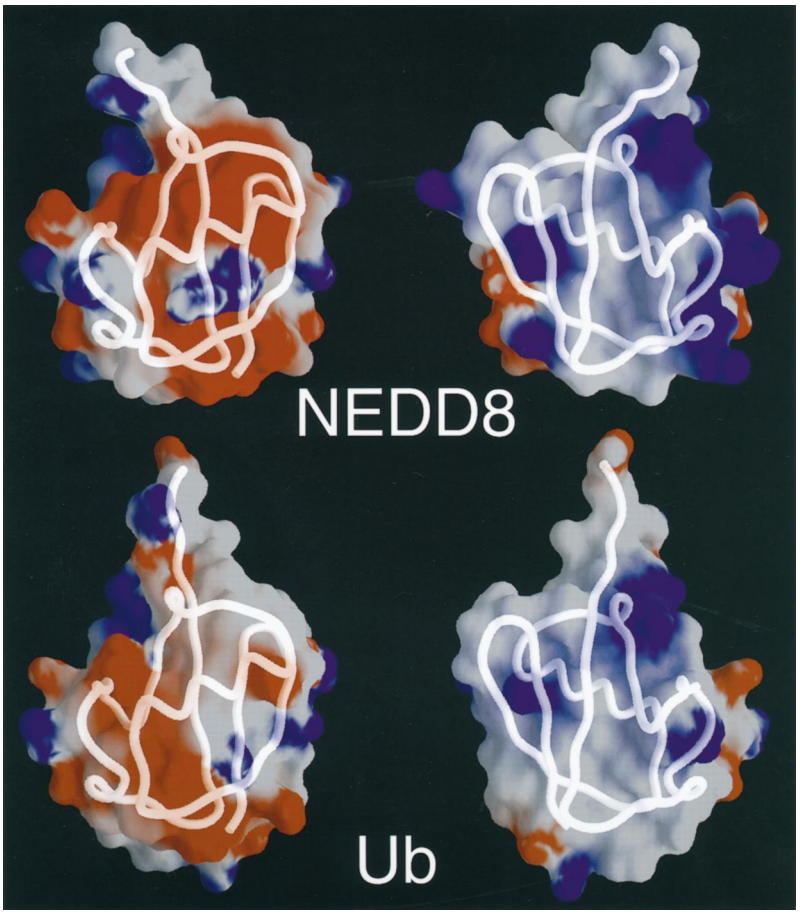

FIG. 5. **Surface electrostatic potential for NEDD8 and Ub.** Two views are shown for NEDD8 (above) and Ub (below). The orientations shown are related by a 180° rotation about the vertical axis, with the acidic face on the *left* and basic face on the *right*. The orientation of the acidic face is very similar to that of Fig. 3. The electrostatic potential is mapped on to the molecular surfaces and is colored in the range −4 kT (*red*) to +4 kT (*blue*). This figure was made with the program GRASP (60).

Leu-8, Ile-44, and Val-70, have been strongly implicated in the interactions of polyUb chains with the 26 S proteasome (33, 46). These side chains are conserved and retain similar levels of solvent accessibility in NEDD8. We compared the solvent accessibilities of other residues in Ub and NEDD8 in an attempt to identify surface exposed hydrophobic residues of NEDD8 that might mediate interactions with specific partner proteins, but no such candidate residues were identified. We note that the side chain of Leu-62 in NEDD8 (Gln in Ub) displays 110 $Å^2$ of solvent accessible surface area. However, this residue is unlikely to mediate a specific protein-protein interaction, since it is poorly conserved among NEDD8 orthologs (see Fig. 4).

Implications of the NEDD8 Structure for Protein-Protein Interactions—The functions of Ub and Ubls are a product of their specific interactions with numerous other proteins, including E1, E2, and E3 enzymes, DUBs, substrate target proteins, and receptor molecules that serve to localize target proteins to specific intracellular locations. Therefore, we compared conserved and divergent residues of Ub and NEDD8 in an effort to identify features of these molecules that may permit them to carry out their evidently distinct biological functions.

Residues that differ between Ub and NEDD8, but whose identity is conserved between the various NEDD8 orthologs, are the best candidates for mediating NEDD8-specific interactions. Seven of these "conserved/divergent" residues are seen in the sequence alignment of Fig. 4. Our data suggest a function for one of these residues, namely Ala-72. Ala-72 is located adjacent to a large area of invariant residues that covers the back and top views in Fig. 6. Residue 72 is Arg in Ub and as such is critical for Ub's interaction with E1 (44). Our observation that NEDD8-A72R binds to E1 with similar affinity as Ub suggests that Ub is likely to contact E1 through a combination of Arg-72 and other residues that are conserved between human NEDD8 and Ub. Arg-42 and Arg-74, which have also been implicated in Ub recognition by E1 (44), are conserved in all of the known NEDD8 orthologs. A fourth residue implicated in the interaction of Ub with E1, Arg-54 (44), is substituted by a similar residue, lysine, in human NEDD8. The interactions of wild-type and R54K-Ub with E1 are indistinguishable from each other.[2] Residue 54 is a leucine in *Arabidopsis* NEDD8; it remains to be determined whether this added divergence (in addition to Ala-72) explains the inability of plant E1 to activate *Arabidopsis* NEDD8 (50).

Our results indicate that Ala-72 plays a key role as a negative specificity determinant in interactions involving NEDD8, by preventing the inappropriate interaction of NEDD8 with E1 and thus with downstream conjugating enzymes. We speculate that Ala-72 is also an important positive determinant of interactions between NEDD8 and the *NEDD8-specific* activating enzyme. This is expected because the Ub E1 enzyme and the Ubl-specific E1-like enzymes show sequence similarity and are thus likely to bind Ub and Ubls in equivalent orientations.

Ala-72 may be a combined positive and negative specificity determinant in deconjugation reactions as well. NEDD8 conjugates persist in mammalian cell extracts under conditions in which endogenous Ub conjugates are completely disassembled, indicating that DUB enzymes act inefficiently on NEDD8 conjugates.[5] The interaction of one DUB enzyme, UCH-L3, with its substrates is predominantly electrostatic (51). The crystal structure of UCH-L3 revealed the presence of multiple anionic residues near the active site, suggesting that basic residues of Ub are involved in substrate binding (52). The recent crystal structure determination of a different UCH enzyme complexed with Ub aldehyde shows that Ub contacts the enzyme almost exclusively through residues that are conserved between NEDD8 and Ub. The sole exception is Arg-72 of Ub, which

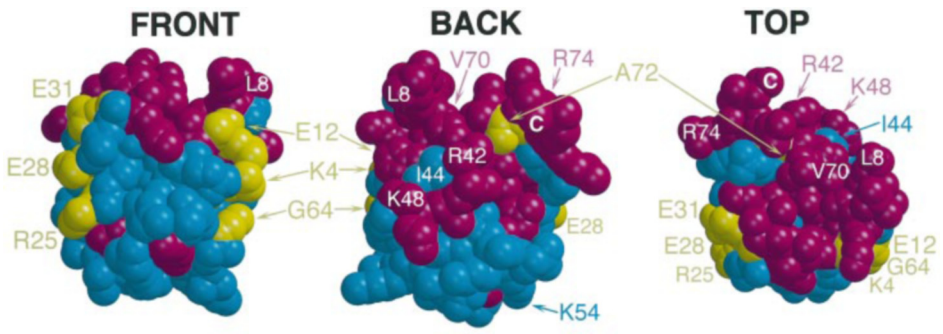

FIG. 6. **Space-filling representation of conserved and divergent residues.** Three views of NEDD8 are shown. The front view is related to the orientation of Fig. 3 by a rotation about the vertical axis of approximately 90° and a twist of approximately 20°. Notice that the residues Glu-31, Glu-28, and Arg-25, are labeled at the side of the front view and are in the center of Fig. 3. The color code is the same as the text background colors of Fig. 4; invariant residues are *magenta*, conserved/divergent residues are *yellow*, and divergent residues are *blue*. The C terminus is labeled with a *C*.

makes a salt bridge interaction with an invariant UCH residue.[6] Furthermore, a mutagenesis study indicates that Arg-72 of Ub makes a significant (1.5 kcal/mol) interaction with UCH-L3.[2]

Strikingly, the other six conserved/divergent residues are arranged in two groups of three adjacent residues that form lines along each side of the NEDD8 molecule as seen in the front view of Fig. 6. Because these two groups of residues are separated from each other by ~25 Å across the molecular surface, it seems likely that they will mediate interactions with different NEDD8-specific partners. Although these residues could mediate interactions with the NEDD8-specific E1-like or DUB-like enzymes, we do not favor this possibility because, as discussed above, we consider it likely that residue 72 and neighboring conserved residues mediate these interactions. A more attractive possibility is that the two stripes of conserved/divergent residues function in the definition of substrate specificity, perhaps through interactions with NEDD8-specific E3-like enzymes. Another attractive possibility is that these residues function in targeting conjugated substrates of NEDD8, such as SCF^{Cdc4}, to defined intracellular locations. NEDD8 and its conjugates are found predominantly in the nucleus (14); it is not known whether NEDD8 conjugates are selectively localized within this compartment.

Lysine residues are of special importance for Ub metabolism by virtue of the proteasomal targeting function of polyUb chains. Most of the lysine residues of Ub are conserved in NEDD8 (see Fig. 4), including Lys-48, which represents the predominant linkage site in polyUb chains (2, 45, 53). At least four other lysine residues can be used in polyUb assembly (3, 54–56), and Lys-63-linked chains have been implicated in mediating a specific DNA repair response (57). Lys-63 is notably absent in NEDD8 and its orthologs (Fig. 4). On the other hand, Phe-4 of Ub is invariably a Lys residue in NEDD8 sequences.

Targeting to the 26 S proteasome is achieved by attachment of a polyUb chain to the substrate. Because efficient targeting requires that the chain contain at least four Ub moieties (35), the crystal structure of tetraubiquitin (Ub_4) has provided a model of the active polyUb conformation (47). One attractive feature of this model is its 2-fold screw axis, which in principle can continue indefinitely to accommodate very long polyUb chains with the same repeating architecture. Another attractive feature is the display of three exposed hydrophobic side chains (Leu-8, Ile-44, Val-70), which have been shown to mediate interactions with the 26 S proteasome, on the surface of the Ub_4 crystal structure. Consistent with the conservation of these residues in NEDD8, we have shown above that a chimeric His_{10}-NEDD8-Ub3 tetramer binds to the 26 S proteasome as tightly as Ub_4.

We have constructed a model for the NEDD8-Ub_3 structure (not shown) by replacement of the most distal Ub moiety in the crystal structure of Ub_4 with an equivalently oriented NEDD8 molecule. This simple modeling exercise suggests that the chimeric construct will be able to adopt a similar conformation as observed in the Ub_4 crystal, since most of the residues that mediate Ub-Ub contacts are conserved (above). A possibly significant exception is Glu-53, which replaces an Ala residue of Ub. The C^{β} atom of Ub Ala-53 is in van der Waals contact with the C^{α} atom of Gly-47 in an neighboring Ub moiety. However, visual inspection by computer graphics suggests that this and other substitutions in NEDD8 can be accommodated by minor rearrangements of the Ub_4 structure. Our model of NEDD8-Ub_3 preserves the chemical character of the putative 26 S proteasome-binding surface of Ub_4; indeed, the putative 26 S proteasome-binding surface is comprised of residues that are largely conserved between NEDD8 and Ub.

Our results show that NEDD8-containing chains are intrinsically capable of functioning in targeting to the 26 S proteasome, but leave open the question of whether such chains ever arise in nature. They are unlikely to be formed via the Ub conjugation system, since NEDD8 not only binds to E1 very weakly, but must also compete with concentrations of Ub that are saturating for E1 (58). NEDD8-containing chains might conceivably arise through the action of the NEDD8-specific activating and conjugating enzymes, which have yet to be analyzed in biochemical detail. Western blot analyses of extracts prepared from mammalian cells revealed an abundant NEDD8 conjugate, which migrated at the position expected for di-NEDD8 (or a chimeric dimer) (14). However, yeast Cdc53, the only NEDD8 substrate identified to date, is modified by just one molecule of NEDD8, and seems to be a stable protein (18). Our data are consistent with this apparent stability; since mono-His_{10}-NEDD8 did not inhibit the degradation of a polyUb-modified test substrate (above), a single NEDD8 is unlikely to be a competent degradation signal. Thus, even if NEDD8 is sometimes assembled into chains and thereby functions in proteolytic signaling, it is also likely to have a distinct biological function.

By analogy to other Ubls, this function probably involves NEDD8 acting as a targeting signal which delivers its substrates to a destination other than the 26 S proteasome. For example, SUMO-1/Smt3 targets certain substrates to the nuclear pore complex (19, 20) or to sites within the nucleus (23), and Ub cross-reactive protein is found on proteins in intermediate filaments of the cytoskeleton (25). The high degree of

[6] S. C. Johnston, S. Riddle, R. E. Cohen, and C. P. Hill, manuscript in preparation.

sequence identity between Ub and all of the NEDD8 orthologs is surprising given that these proteins apparently participate in many distinct intermolecular interactions. The high degree of similarity may have been maintained through a requirement to bind common partners, although this idea must be considered speculative. Our results indicate that NEDD8 retains many determinants of Ub's proteolytic signaling function. Therefore it is not surprising that at least one functionally significant mutation (Arg-72 of Ub to Ala-72 of NEDD8) served to eliminate a positive determinant of Ub function. Future studies should reveal whether this mutation simultaneously created a positive determinant of NEDD8 function, and whether any of the other conserved/divergent residues play dual roles.

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