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Structural Basis for Recruitment of Ubc12 by an E2 Binding Domain in NEDD8's E1

Danny T. Huang,[1] Amir Paydar,[1] Min Zhuang,[1,2] M. Brett Waddell,[1] James M. Holton,[3] and Brenda A. Schulman[1,4,*]
[1]Department of Structural Biology
Department of Genetics/Tumor Cell Biology
St. Jude Children's Research Hospital
Memphis, Tennessee 38105
[2]Interdisciplinary Program
University of Tennessee Health Sciences Center
Memphis, Tennessee 38163
[3]Physical Biosciences Division
Lawrence Berkeley National Laboratory
University of California, Berkeley
Berkeley, California 94720
[4]Department of Molecular Sciences
University of Tennessee Health Sciences Center
Memphis, Tennessee 38163

Summary

E2 conjugating enzymes play a central role in ubiquitin and ubiquitin-like protein (ublp) transfer cascades: the E2 accepts the ublp from the E1 enzyme and then the E2 often interacts with an E3 enzyme to promote ublp transfer to the target. We report here the crystal structure of a complex between the C-terminal domain from NEDD8's heterodimeric E1 (APPBP1-UBA3) and the catalytic core domain of NEDD8's E2 (Ubc12). The structure and associated mutational analyses reveal molecular details of Ubc12 recruitment by NEDD8's E1. Interestingly, the E1's Ubc12 binding domain resembles ubiquitin and recruits Ubc12 in a manner mimicking ubiquitin's interactions with ubiquitin binding domains. Structural comparison with E2-E3 complexes indicates that the E1 and E3 binding sites on Ubc12 may overlap and raises the possibility that crosstalk between E1 and E3 interacting with an E2 could influence the specificity and processivity of ublp transfer.

Introduction

Posttranslational modifications by ubiquitin and ublps such as NEDD8, ISG15, and SUMO control a vast array of biological processes including the cell cycle, development, inflammation, and the immune response (Schwartz and Hochstrasser, 2003). Defects in these pathways have been associated with diseases such as cancer, neurodegenerative disorders, and viral infections (Ciechanover and Schwartz, 2004). Unlike ubiquitin, the ublp NEDD8 does not direct its targets for proteasomal degradation. Instead, NEDD8 modifies the cullin subunits of Skp1-cullin-F-box (SCF) and other cullin-based ubiquitin ligases to regulate their enzymatic activity. NEDD8 boosts the ability of SCFs to polyubiquitinate their targets and prevents binding of the SCF inhibitor CAND1 (Pan et al., 2004). The NEDD8 pathway regulates many important biological processes, including cell division, signal transduction, and development,and is essential for viability of a range of organisms, including mammals (Pan et al., 2004).

Ubiquitin and the ublps NEDD8, ISG15, and SUMO are ligated to their targets by parallel cascades involving a series of three enzymes in classes known as E1, E2, and E3 (Hershko et al., 1983; Pickart and Eddins, 2004). The sequences of E1s and E2s and some E3s from each pathway display common features, suggesting that common mechanisms underlie posttranslational modification by ubiquitin and these ublps. A ublp's dedicated E1 initiates the cascade by catalyzing adenylation of the ublp's C terminus. After this activation reaction, the E1 catalyzes a second reaction to form a covalent thioester intermediate between the E1's catalytic cysteine and the ublp's C terminus. The E1 binds an E2 and then catalyzes a third chemical reaction, promoting transfer of the ublp to the E2's catalytic cysteine. The product of this third E1-catalyzed reaction is a covalent E2∼ublp thioester intermediate. Transfer of the ublp from the E2 to the target is often facilitated by an E3. In general, E3s have at least two domains: (1) a domain, often a RING, HECT, or related motif that recruits the E2, and (2) a protein-protein interaction domain that recruits the target. Therefore, E2s are central to the relay—accepting the ublp from the E1 and coordinating the ublp with downstream steps in the cascade.

NEDD8's dedicated E2, Ubc12, interacts with NEDD8's E1 in a distinct, bipartite manner (Huang et al., 2004; VanDemark and Hill, 2004). Ubc12 contains a unique N-terminal, 13 residue peptide-like sequence that docks in a groove unique to NEDD8's E1, distal from the E1's catalytic cysteine (Huang et al., 2004). In addition, Ubc12 also interacts with NEDD8's E1 through its ∼150 residue catalytic core domain (Huang et al., 2004). The catalytic core domain is the common feature of E2s for different ublps (Pickart and Eddins, 2004). Although several ubiquitin E2s contain extensions at the N-, C-, or N- and C termini beyond the core domain, many E2s for ubiquitin, the E2 for SUMO (Ubc9), and an E2 for ISG15 (UbcH8) consist exclusively of a catalytic core domain (Kim et al., 2004; Pickart and Eddins, 2004; Zhao et al., 2004). Thus, a catalytic core domain is minimally sufficient for interacting with E1, forming an E2∼ublp thioester intermediate, interacting with E3-target complexes, and transferring the ublp. Detailed structural studies have revealed the bases for many of these E2 activities, including an E2's interactions with ubiquitin in a thioester complex (Hamilton et al., 2001), interactions between E2s and E3s (Dominguez et al., 2004; Huang et al., 1999; Zheng et al., 2000), and an E2's interaction with a target (Bernier-Villamor et al., 2002). However, detailed knowledge of E1-E2 contacts is also critical for understanding the central function of E2s in ublp transfer cascades. To address this problem, we report here the crystal structure of the complex between the C-terminal ubiquitin-fold domain of NEDD8's E1 and the core domain of Ubc12.

*Correspondence: brenda.schulman@stjude.org

Results and Discussion

The NEDD8 E1's C-Terminal Ubiquitin-Fold Domain Binds to Ubc12's Core Domain

Previous studies of NEDD8's heterodimeric E1, the APPBP1-UBA3 complex, and NEDD8's E2, Ubc12, suggested a bipartite E1-E2 interaction. The crystal structure of NEDD8's E1 in complex with a peptide corresponding to Ubc12's N-terminal extension showed Ubc12's N-terminal 13 residues binding a groove unique to NEDD8's E1, distal from the E1's active site cysteine (Huang et al., 2004). However, it remained unknown as to where Ubc12's core domain binds the E1. Crystal structures of the E1 revealed three domains: an adenylation domain containing the ATP binding site is linked through flexible loops to one domain organized around the catalytic cysteine and to another domain at the C terminus of the UBA3 subunit that adopts a structure similar to ubiquitin and ublps (Huang et al., 2004; Walden et al., 2003a, 2003b). Deletion of the E1's C-terminal ubiquitin-fold domain reduced the ability of the E1 to bind Ubc12 and to promote Ubc12~NEDD8 thioester formation (Walden et al., 2003b). This prompted us to test whether the E1's ubiquitin-fold domain recruits Ubc12's core domain to form the second portion of the bipartite E1-Ubc12 interaction. We examined E1-mediated transfer of ^{32}P-labeled NEDD8 onto the catalytic cysteine of a fragment of Ubc12 corresponding to the core domain (previously referred to as Ubc12ΔN [Huang et al., 2004], but referred to in this work as Ubc12^{core}). With the wild-type E1, the Ubc12^{core}~^{32}P-NEDD8 complex is readily detectable (Figure 1A). However, with the E1 deletion mutant lacking the C-terminal ubiquitin-fold domain, Ubc12^{core}~^{32}P-NEDD8 thioester formation is detectable only with the highest concentration of Ubc12^{core} we can generate and only after an extended reaction time (Figure 1A).

Additional data support the notion that the E1's C-terminal ubiquitin-fold domain interacts directly with Ubc12's core domain. First, a fragment of the *N*EDD8 *E1* corresponding to the *u*biquitin-*f*old *d*omain (UBA3 residues 347–442, hereafter referred to as NE1^{ufd}) binds Ubc12^{core} in a nondenaturing mobility shift assay (Figure 1B). Because the pI of NE1^{ufd} is ~7.2, it migrates slowly in a pH 8.0 nondenaturing gel. By contrast the pI of Ubc12^{core} is ~5.2, so it migrates more rapidly. Mixing NE1^{ufd} with Ubc12^{core} leads to the disappearance of the bands for each individual protein and to the appearance of a new band of intermediate migration corresponding to the NE1^{ufd}-Ubc12^{core} complex (Figure 1B). Second, addition of the isolated NE1^{ufd} fragment to a transthiolation assay inhibits the E1-mediated formation of the Ubc12~NEDD8 thioester complex (Figure 1C). Taken together, these results suggest that the second portion of the bipartite E1-Ubc12 interaction is mediated by the E1's ubiquitin-fold domain and Ubc12's catalytic core domain. The secondary structure propensities and the pattern of hydrophobic and hydrophilic residues are conserved among sequences at the C termini of other E1s and among E2s, reflecting conservation of their folds (Figures 2A and 2B). Thus, it is likely that other E1-E2 interactions will resemble those between NE1^{ufd}-Ubc12^{core}.

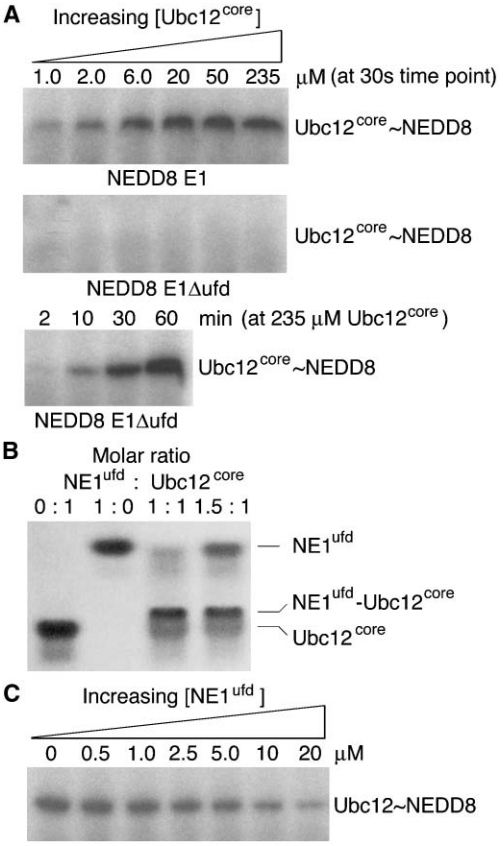

Figure 1. The NEDD8 E1's C-Terminal Ubiquitin-Fold Domain Binds Ubc12's Core Domain

(A) Deletion of the C-terminal ubiquitin-fold domain from the UBA3 subunit of NEDD8's E1 diminishes Ubc12^{core}~NEDD8 thioester formation. 30 s time points of transthiolation reactions were performed with ^{32}P-NEDD8, increasing concentrations of Ubc12^{core} as indicated, and the full-length NEDD8 E1 (top) or the NEDD8 E1Δufd, lacking UBA3 residues 348–442 (middle). Bottom: time course of transthiolation with ^{32}P-NEDD8, the highest concentration of Ubc12^{core} used in the upper panels (235 μM), and the NEDD8 E1Δufd. Ubc12^{core}~^{32}P-NEDD8 was visualized by autoradiography after SDS-PAGE.

(B) Protein-protein interactions observed in a nondenaturing polyacrylamide gel mobility shift assay with the indicated ratios of NE1^{ufd}:Ubc12^{core} visualized by Coomassie blue staining. Migration of Ubc12^{core} is retarded by complex formation with NE1^{ufd}.

(C) E1-catalyzed Ubc12~^{32}P-NEDD8 thioester formation examined in the presence of increasing concentrations of the isolated NE1^{ufd}. The NE1^{ufd} inhibits the reaction.

Overall Structure of the NE1^{ufd}-Ubc12^{core} Complex: A Common Mechanism for E1-E2 Interactions

In order to understand how NEDD8's E1 recruits Ubc12's core domain, we determined the structure of the NE1^{ufd}-Ubc12^{core} complex at 2.4 Å resolution (Table 1). The structure of the NEDD8 E1's ubiquitin-fold domain has been described previously and adopts an α/β structure similar to ubiquitin and other ublps (Figures 2A and 2C) (Walden et al., 2003b). The two major significant differences between the overall structures of NE1^{ufd} and ubiquitin are: (1) the C-terminal strand of NE1^{ufd} (βo) corresponds to the N-terminal strand in ubiquitin but is in the opposite orientation, and (2) a kinked α helix (α13) is inserted between β strands m and n of NE1^{ufd}. One face

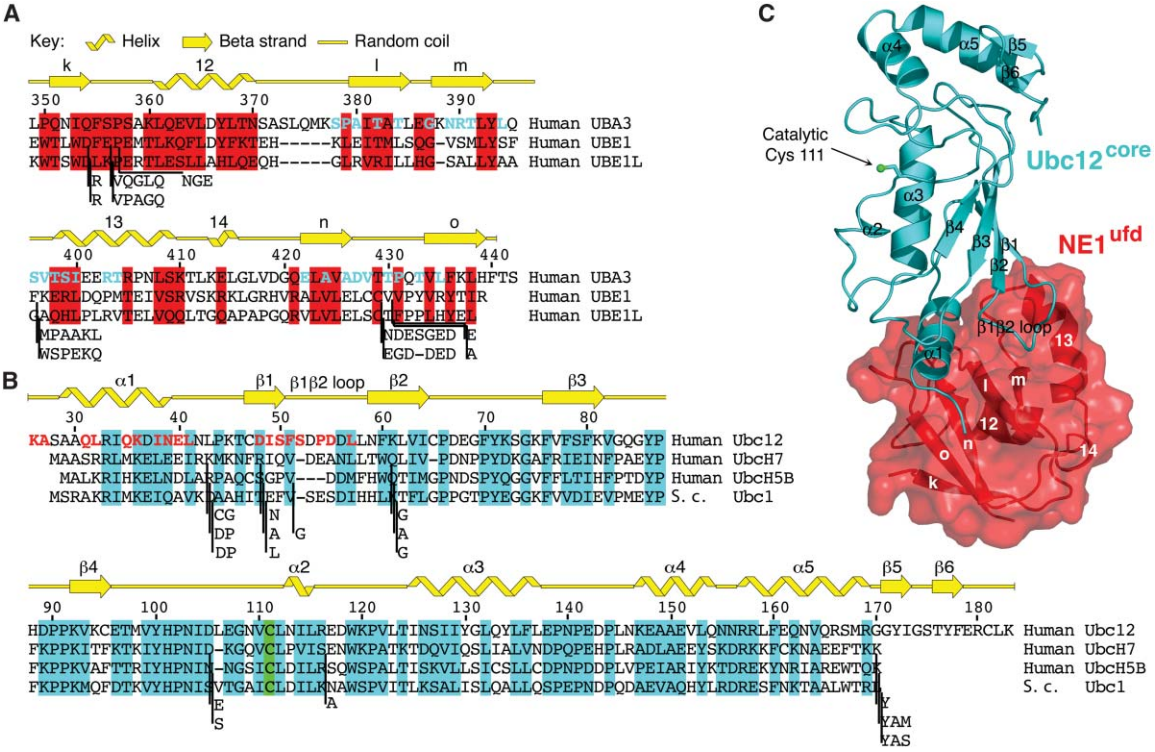

Figure 2. Overall Structure of the NE1^{ufd}-Ubc12^{core} Complex

(A) Sequence alignment of C-terminal ubiquitin-fold domains from human UBA3 (subunit of the E1 for NEDD8), human UBE1 (E1 for ubiquitin), and human UBE1L (E1 for ISG15). Secondary structure elements are indicated above the alignment. Residues of identical nature (either hydrophobic or hydrophilic) in all three proteins reflect common structure propensities and are highlighted in red. Residues in UBA3's ubiquitin-fold domain (NE1^{ufd}) that contact Ubc12^{core} are colored cyan. Sequences of insertions in the ubiquitin-fold domains from UBE1 and UBE1L are indicated below.

(B) Sequence alignments of human Ubc12's core domain and three E2s for ubiquitin, human UbcH7, human UbcH5B, and *S. cerevisiae* Ubc1, with secondary structure elements indicated above and insertions in the sequences of the ubiquitin E2s indicated below. Residues of identical nature (either hydrophobic or hydrophilic) in all three proteins reflect common structure propensities and are highlighted in cyan, and the location of Ubc12's catalytic Cys111 is highlighted in green. Residues in Ubc12^{core} that contact NE1^{ufd} are colored red.

(C) Overall structure of the complex, with NE1^{ufd} in red cartoon representation overlaid with a transparent surface and Ubc12^{core} shown in cyan cartoon. Secondary structures are labeled, and the position of Ubc12^{core}'s catalytic cysteine is represented by a green sphere. Figures were made using Pymol (DeLano).

of the domain is formed by α13 and four strands of the five-stranded, twisted antiparallel β sheet. The other face is formed by the fifth strand (βk) and the α12 helix conserved between the structures of NE1^{ufd} and ubiquitin.

Ubc12^{core} adopts the canonical E2 core domain fold (Cook et al., 1992) (Figures 2B and 2C). Two regions of Ubc12^{core} differ significantly from the structures of E2s for ubiquitin and SUMO (Cook et al., 1992; Giraud et al., 1998; Tong et al., 1997). First, Ubc12^{core}'s N-terminal α1 helix is one turn shorter at its C terminus, a feature that may contribute to specificity of E1-E2 interactions (see below). Second, the C-terminal α5 helix is also shorter, and it extends into a two-stranded antiparallel β sheet not yet found in any other E2. This sheet makes hydrophobic interactions with a surface formed by the two C-terminal helices and the loop after β4. This loop that packs against Ubc12's β sheet contains the "HPN" motif that includes a catalytically important asparagine found in all E2s (Wu et al., 2003b).

The overall structure of the NE1^{ufd}-Ubc12^{core} complex adopts a compact, globular ovoid shape, resembling an extended version of the already oblong E2 fold (Figure 2C). Four of the five β strands and the kinked α13 helix of NE1^{ufd} form a W-shaped surface, which recruits the N-terminal end of Ubc12^{core}. The W-shaped surface is generated from two V-shaped grooves. One of the NE1^{ufd} V-shaped grooves consists of β strands l, m, n, and o. This groove cradles Ubc12's long α1 helix along its entire length, which lies parallel to the overall direction of the strands in NE1^{ufd}. The second NE1^{ufd} V-shaped groove has β strand m as its base and βl and the kinked α13 helix as its sides, and also holds Ubc12's β1β2 loop. The entire NE1^{ufd}-Ubc12^{core} interaction buries 943 Å^2 of exposed surface area from NE1^{ufd} and 898 Å^2 from Ubc12^{core}. NE1^{ufd} binds the opposite face of Ubc12^{core} from the catalytic cysteine, perhaps to present Ubc12's active site to the E1's from a distance.

Several previous studies suggest that E2s for other ublps bind their E1s in a similar manner. Two mutational studies of ubiquitin E2s showed a role for the N-terminal helix in E2∼ubiquitin thioester formation (Pitluk et al., 1995; Sullivan and Vierstra, 1991). Although these studies did not directly assay E1 binding, they led to the

Table 1. Data Collection and Refinement Statistics

Selenomethionine NE1^{ufd}-Ubc12^{core} MAD Data Analysis			
	Peak	Inflection	Remote
Wavelength (λ)	0.9790	0.9792	0.95
Resolution (Å)	50.0–2.4	50.0–2.4	50.0–2.4
Total reflections	402,132	399,152	397,209
Unique reflections	12,655	12,967	12,769
Overall R_{sym} (%)	9.9 (37.8)	9.0 (38.4)	9.0 (38.6)
Overall I/σ (I)	38.3 (10.4)	37.0 (8.8)	36.8 (8.8)
Overall Figure of Merit 0.59			
Data from Peak Wavelength			
Space group	$P2_12_12_1$		
Cell dimensions			
a, b, c (Å)	40.6, 61.5, 125.9		
α, β, γ (°)	90, 90, 90		
Completeness (%)	99.3 (99.9)		
Mean redundancy	11.4		
Refinement Statistics			
R_{work}/R_{free} (%)	24.2/25.9		
Number of atoms			
Protein	1996		
Water	112		
Selenium	4		
Rms deviations			
Bond lengths (Å)	0.007		
Bond angles (°)	1.51		
Average B factor	44.0		

Numbers in parentheses are for the high-resolution bin. $R_{sym} = |I - \langle I \rangle|/I$, where I is the intensity of an individual measurement, and $\langle I \rangle$ is the average intensity from multiple observations. $R_{work} = ||Fobs| - k|Fcalc||/|Fobs|$. R_{free} equals the R_{work} against 5% of the data removed prior to refinement.

hypothesis that the N-terminal helix of ubiquitin E2s is involved in binding to E1 (Cook et al., 1992). In addition, mutation of the N-terminal helix and β1β2 loop in SUMO's E2 diminishes binding to SUMO's E1 (Bencsath et al., 2002). Interestingly, a bioinformatics study found that the N-terminal helix is one of the most evolutionarily conserved regions among E2s for a particular ublp (Winn et al., 2004). Moreover, E2s for different ublps have their own distinguishing N-terminal helix motifs (Winn et al., 2004).

Details of the Interface between the NEDD8 E1's Ubiquitin-Fold Domain and Ubc12^{core}

Formation of the NE1^{ufd}-Ubc12^{core} complex is driven by two sets of hydrophobic interactions (Figure 3A). The smaller hydrophobic cluster involves NE1^{ufd}'s Ala424, Ala426, Thr433, and Leu435, and Ubc12's Ala27 and Leu32 from the N-terminal α1 helix. The second, larger hydrophobic cluster involves NE1^{ufd}'s Thr382 and Thr391's methyl groups, SeMet394 (in our structure, Leu394 in wild-type Ubc12), Val397, and Ile400 packing against Ubc12^{core}'s Ile38 and Leu41 from the C-terminal end of the α1 helix, and Ile49, Phe51, and Leu57 from the β1β2 loop.

The NE1^{ufd}-Ubc12^{core} interaction is also stabilized by numerous hydrogen bonds and salt bridges: between the E1's Thr382 and Ubc12's Gln35, between the E1's Arg390 and Ubc12's Asp55, and between the E1's Ser396 and Ubc12's Asn39. Ubc12's conserved Lys36 interacts with a backbone oxygen in the turn between NE1^{ufd}'s β strands n and o. Interestingly, the NE1^{ufd}-Ubc12^{core} interface parallels ubiquitin's interactions with its partner proteins: Thr382, Ala426, and Thr433 correspond to ubiquitin's Ile44, Val70, and Leu8, respectively, which make important contacts to most ubiquitin binding proteins (Beal et al., 1996; Sloper-Mould et al., 2001) (see below).

Sequence analysis suggests that the nature of interactions between E2s with the ubiquitin-fold domains of their E1s is preserved throughout evolution. Many residues critical for complex formation are conserved in NEDD8's E1 and Ubc12 family members. Leu394, Val397, Ile400, and Ala424 are conserved as hydrophobics, and Thr382 and Arg390 are conserved as polar or charged residues in NEDD8 E1 orthologs from yeast to man. From Ubc12s, Leu32, Ile38, Leu41, Phe51, and Leu57 are conserved as hydrophobic residues, with Gln35, Lys36, and Asp55 conserved as polar or charged residues throughout evolution. In addition, the sequences of E1s and E2s for different ublps appear to have coevolved to maintain analogous E1-E2 interactions. Differences between the sequences of E1s for NEDD8 and ubiquitin correlate with differences in the corresponding interaction regions of their E2s. One example is Ubc12's highly conserved Leu32, which interacts with a hydrophobic patch on NE1^{ufd} that includes Ala424 and Ala426. By contrast, Leu32 is replaced by a basic arginine or lysine in the sequences of E2s for ubiquitin, and the sequences of E1s from different organisms have an acidic aspartate or glutamate in place of either the NEDD8 E1's Ala424 or Ala426. Another difference between Ubc12 and E2s for ubiquitin is at

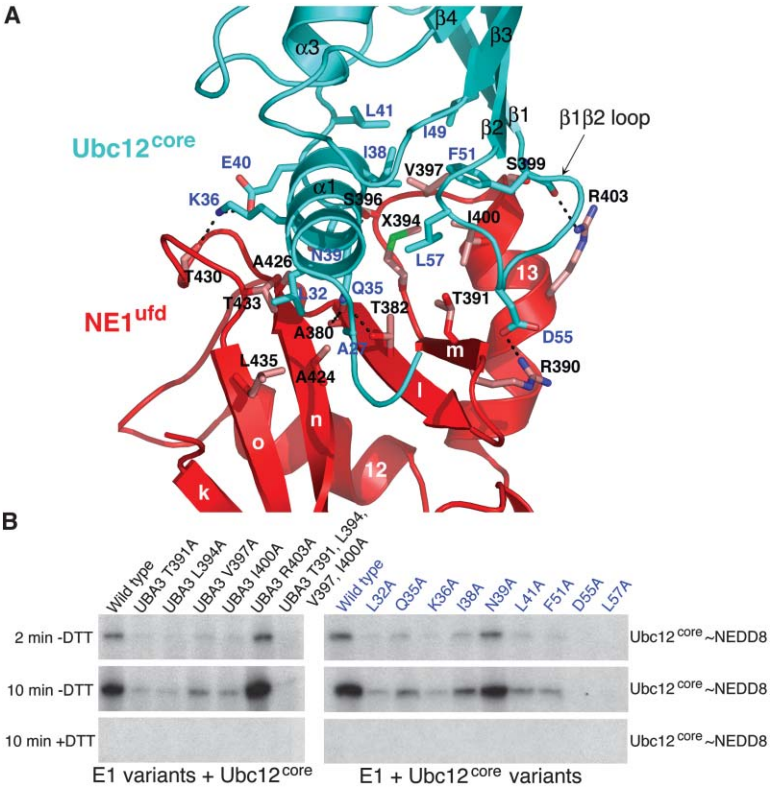

Figure 3. Details of the NE1ufd-Ubc12core Interface

(A) Closeup view of interactions between NE1ufd and Ubc12core. NE1ufd is shown in red. NE1ufd's side chains are shown in pink and labeled in black. Ubc12core is shown in cyan. Ubc12core's side chains are shown in cyan and labeled in blue. Oxygen atoms are colored red, nitrogen atoms blue, and selenium green. Hydrogen bonds are represented with dashes. Side chains are labeled with single-letter code, with X for selenomethionine.

(B) Formation of the Ubc12core~32P-NEDD8 thioester complex was assayed at 2 and 10 min as indicated, resolved by SDS-PAGE, and visualized by autoradiography. The thioester bond is confirmed by susceptibility to reduction by DTT at the final time point. Wild-type and mutant versions of NEDD8's E1 (left, with wild-type Ubc12core) or of Ubc12core (right , with the wild-type E1) used in each reaction are denoted above each lane.

the C terminus of α1, which is one turn shorter in Ubc12 than in E2s for ubiquitin. An additional turn of the α1 helix would clash with the structure of NE1ufd, preventing a ubiquitin E2 from forming an incorrect complex with NEDD8's E1. The region of the ubiquitin E1 predicted to contact the C terminus of the α1 helix differs completely from the sequences found in NEDD8 E1s but is highly conserved among E1s for ubiquitin. Thus, although E1s for other ublps are likely to bind their E2s through parallel surfaces, specific E1-E2 interactions likely distinguish the pathways for different ublps.

To confirm the relevance of the contacts observed in our structure, we tested the effects of mutating interface residues to alanines on E1-mediated transfer of NEDD8 onto Ubc12core (Figure 3B). There are deleterious effects of mutating the Thr391, Leu394, Val397, and Ile400 anchor side chains from NEDD8 E1's ubiquitin-fold domain, while there is no significant effect of mutating Arg403, which contacts the Ubc12 backbone. A quadruple mutant, with alanine substitutions for the four anchor residues, is significantly impaired for Ubc12core~NEDD8 thioester formation. We also tested the effects of alanine substitutions in Ubc12core (Figure 3B). Mutation of the following key interface residues significantly diminishes formation of the Ubc12core~NEDD8 thioester complex: Leu32, Gln35, Lys36, Ile38, Leu41, Phe51, Asp55, and Leu57. These results indicate that the interactions observed in the NE1ufd-Ubc12core crystal structure are important for the transthiolation reaction.

Rotation of the NEDD8 E1 Ubiquitin-Fold Domain for the Transthiolation Reaction

To gain insight into NEDD8 transfer from E1 to E2, we superimposed the NE1ufd-Ubc12core structure onto the structure of the C-terminal ubiquitin-fold domain in the previous E1 structures, including a structure of NEDD8's E1 in complex with a peptide corresponding to Ubc12's N-terminal extension (Figure 4A) (Huang et al., 2004; Walden et al., 2003a, 2003b). Although we do not know whether the orientation of the ubiquitin-fold domain observed in the previous structures is physiologically significant, the domain is in a similar location in multiple different crystal forms, suggesting that the crystallized conformation is populated under some conditions. The superposition agrees with the previous estimate of 28 Å for the distance between the C-terminal residue visible in the structure of Ubc12's N-terminal peptide (residue 13) and the N terminus of Ubc12core (residue 27) that was based on the kinetic effects of deletions within the linker connecting Ubc12's N-terminal extension and core domain (Huang et al., 2004). However, in the models of a complex, Ubc12core's active site cysteine faces the opposite direction from E1's large central groove containing the NEDD8 binding site and the E1's catalytic cysteine. Moreover, a greater than 50 Å gap separates the E1 and E2 catalytic cysteines in the model (Figure 4A). This raises the possibility that a significant conformational change would be required for juxtaposition of the E1's and Ubc12's active site cysteines during the transthiolation reaction. Clues to a possible solution to this problem come from previous studies of a ubiquitin E2′s interactions with HECT domain E3s. A similarly large gap was observed between the active site cysteines in the complex between UbcH7 and the HECT domain of E6AP (Huang et al., 1999). In a subsequent structure of the HECT domain from WWP1, the lobe containing the catalytic cysteine is rotated ~100° about the linker to the E2 binding lobe (Verdecia et al., 2003).

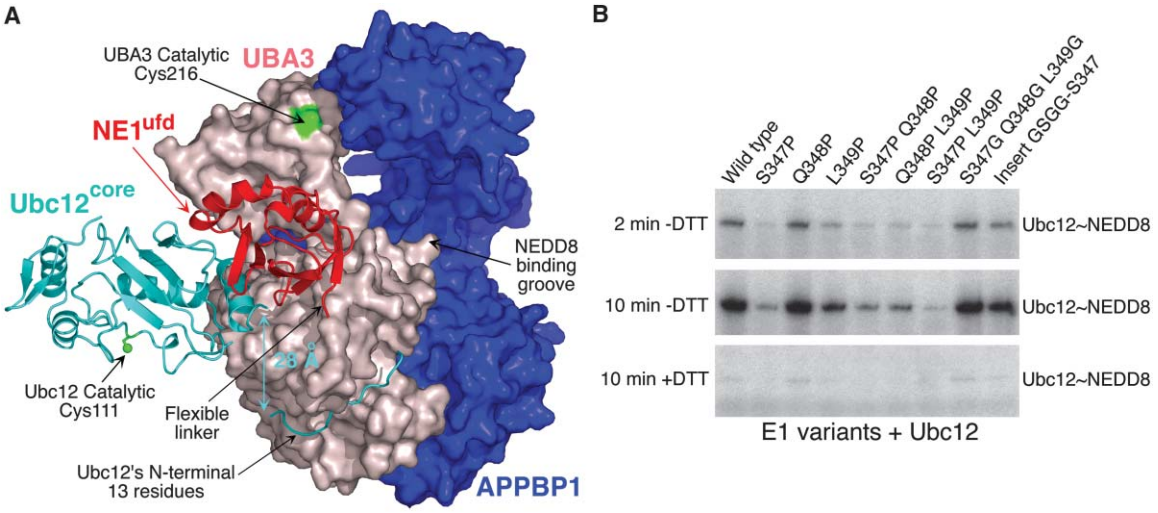

Figure 4. Evidence for Rotation of the E1's Ubiquitin-Fold Domain in Ubc12∼NEDD8 Thioester Formation

(A) Superposition of the NE1^{ufd}-Ubc12^{core} complex structure with the previous structure of the complex between NEDD8's E1 and the Ubc12N26 peptide (Huang et al., 2004), generated by least-squares alignment of all Cα atoms in the E1's ubiquitin-fold domain that is common to both structures (Jones et al., 1991). The APPBP1 subunit of NEDD8's E1 is depicted as a blue surface, and the UBA3 portions of the adenylation and catalytic-cysteine-containing domains are shown as pink surfaces with the catalytic cysteine in green. UBA3's C-terminal ubiquitin-fold domain is a red cartoon. Both Ubc12's N-terminal extension, from the E1 complex with Ubc12N26 (Huang et al., 2004), and Ubc12^{core} are shown in cyan ribbon. Ubc12's catalytic cysteine is represented as a green sphere. The location of the NEDD8 binding groove within the E1 structure is denoted "NEDD8 binding groove." The location of the linker connecting the ubiquitin-fold domain with the rest of the E1 is denoted "flexible linker." The distance between the C-terminal residue of Ubc12's N-terminal extension (residue 13) and the N terminus of Ubc12^{core} (residue 27) visible in the crystal structures is 28 Å, consistent with previous data (Huang et al., 2004). Ubc12's catalytic cysteine faces the opposite direction and is 50 Å away from the E1 catalytic cysteine in UBA3 in the structural superposition.

(B) Mutational analysis of the linker connecting the ubiquitin-fold domain to the remainder of the E1. Thioester formation between Ubc12 and ^{32}P-NEDD8 was assayed at 2 and 10 min as indicated, resolved by SDS-PAGE, and visualized by autoradiography. The thioester complex is confirmed by susceptibility to reduction by DTT at the final time point. Wild-type, proline, and glycine mutant versions of NEDD8's E1 used in each reaction are denoted above each lane.

This rotation would bring the E2's and E3's catalytic cysteines closer together. It is thought that the domain rotation in HECT E3s allows successive cycles of ubiquitin loading and unloading for polyubiquitin chain formation (Verdecia et al., 2003).

We wondered whether the linker connecting the E1's C-terminal ubiquitin-fold domain to the remainder of the E1 undergoes an analogous rotation during the transthiolation reaction. Indeed, four aspects of the previous full-length NEDD8 E1 structures are consistent with the possibility that the ubiquitin-fold domain could rotate relative to the remainder of the E1 structure. First, a comparison between different complexes formed by NEDD8's E1 revealed rotations about the linkers connecting the different domains, raising the potential for domain rotations to play a role in catalysis (Walden et al., 2003a). Second, in previous structures of the full-length NEDD8 E1, the three residues in the linker, Ser347, Gln348, and Leu349, make no specific side chain contacts to the remainder of the E1 structure (Huang et al., 2004; Walden et al., 2003a, 2003b). Third, both isolated NE1^{ufd} alone, and a truncated version of the E1 lacking the ubiquitin-fold domain, are well behaved, independently folded units. Fourth, in the previous structures of the full-length NEDD8 E1, the electron density is weak and B factors are very high for the ubiquitin-fold domain, consistent with the possibility that this domain is particularly mobile (Huang et al., 2004; Walden et al., 2003a, 2003b).

We tested the functional importance of flexibility in the linker with mutations to prolines or glycines: mutation to proline restricts rotation about the polypeptide backbone, whereas mutation to glycine allows the greatest conformational freedom (Ramachandran and Sasisekharan, 1968). Mutation of Ser347 or Leu349 to proline impairs E1-mediated Ubc12∼NEDD8 thioester formation, as does any pair of proline mutations in the linker (Figure 4B). By contrast, mutation of the linker residues to glycines or insertion of the flexible sequence Gly-Gly-Ser-Gly has little effect on Ubc12∼NEDD8 thioester formation (Figure 4B). Thus, the ability of the linker to rotate, irrespective of its exact sequence, may be important for NEDD8 transfer from E1 to Ubc12.

Large conformational changes are a common feature among enzymes involved in ublp transfer. Although the best characterized conformational changes accompany polyubiquitination by the HECT class of E3 ligases (Verdecia et al., 2003), there are several other enzymes for which requirements for large conformational changes can be inferred from large distances between catalytic sites observed in crystal structures. For example, after NEDD8 adenylation, the E1's catalytic cysteine attacks the activated NEDD8 C terminus to form the covalent E1∼NEDD8 thioester complex. However, in the structure of the E1-NEDD8-ATP complex, NEDD8's C terminus is ∼30 Å away from the active site cysteine involved in formation of the thioester intermediate (Walden et al., 2003a). Thus, there appears to be a requirement for

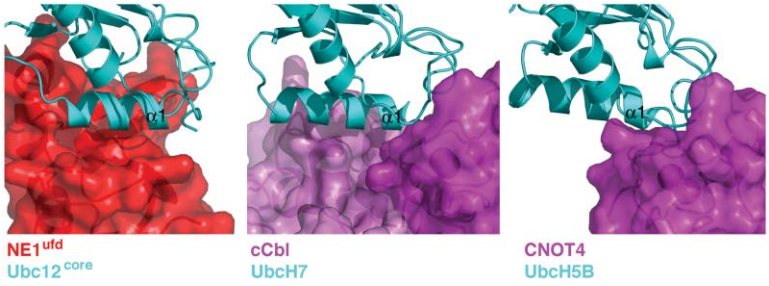

Figure 5. Recognition of Common E2 Structural Elements by NE1^{ufd} and E3s

Structures of NE1^{ufd} in complex with the E2 Ubc12^{core}, the E3 c-Cbl in complex with the E2 UbcH7 (Zheng et al., 2000), and the RING domain of the E3 CNOT4 in complex with the E2 UbcH5B (Dominguez et al., 2004), shown from left to right, with UbcH7 and UbcH5B in the same orientation as Ubc12^{core}. The E2 binding domain of the E1, NE1^{ufd}, is shown in red, and of the E3s, c-Cbl and CNOT4, are shown in magenta. The E2s Ubc12^{core}, UbcH7, and UbcH5B are shown in cyan. The corresponding regions of the first turn of the N-terminal helix in Ubc12^{core}, UbcH7, and UbcH5B are labeled "α1," and are involved in binding to NE1^{ufd}, c-Cbl, and CNOT4, respectively.

rotation of the C terminus of NEDD8 and/or the E1's catalytic cysteine-containing domain in forming the E1∼NEDD8 thioester intermediate. Structural data also suggest 30–50 Å distances between the E2 active site cysteine and the target lysine in SCF ubiquitin ligase complexes (Orlicky et al., 2003; Wu et al., 2003a). Perhaps this distance in SCFs allows for loading and unloading successive ubiquitin molecules in a polyubiquitin chain, as is thought to be the case with HECT E3s (Verdecia et al., 2003).

Although it is not yet clear how and why enzymes in ublp transfer cascades undergo conformational changes, analogous large-scale motions in other classes of enzymes may shed light on these questions. Several multifunctional biosynthetic enzymes, such as acetyl-coenzymeA synthetase and nonribosomal peptide synthetases, also appear to undergo dramatic domain rotations to carry out successive catalytic steps in an assembly line manner (Challis and Naismith, 2004). In reactions akin to E1's, these multidomain enzymes first activate their substrates by adenylation and then catalyze transfer of their activated substrates to a nucleophilic thiol at the terminus of coenzyme A or phosphopantetheine. Structures of acetyl-coenzymeA synthetase and non-ribosomal peptide synthetase enzymes revealed that the different catalytic active sites are located far apart, and in different domains, of these modular enzymes (Conti et al., 1997; Gulick et al., 2003). An ∼140° rotation about the linker between the adenylation domain and the C-terminal domain in acetyl-coenzymeA synthetase brings the active site for the second reaction into close proximity with its substrate, which is the product of the first reaction (Gulick et al., 2003). Completion of each step in the series of reactions is thought to cause a domain rotation necessary for catalyzing the next step. It is plausible that the enzymes that catalyze ublp transfer function in an analogous manner, with each reaction triggering a conformational change necessary for the next step in the cascade.

Implications for E1-E2-E3 Transfer Cascade

The ultimate function of Ubc12 is to ligate NEDD8 to lysine side chains of target proteins. The best-characterized targets of NEDD8 are cullin family members (Pan et al., 2004). NEDD8 modification of cullins requires interaction between the E2, Ubc12, and the RING family E3, Rbx1 (also known as Roc1 or Hrt1), and mutational studies suggest that the Rbx1/Roc1/Hrt1 E3 recruits Ubc12 in a manner resembling other RING E3-E2 complexes (Kamura et al., 1999; Morimoto et al., 2003). There is presently no structure of a Ubc12-Rbx1/Roc1/Hrt1 complex. However, structural data are available for two E2-RING E3 complexes in the ubiquitin pathway: between the E2 UbcH7 and the E3 c-Cbl and between the E2 UbcH5B and the RING domain of the E3 CNOT4 (Dominguez et al., 2004; Zheng et al., 2000). Although it is not known whether any E1s and E3s bind to the same site on E2s, comparison of NE1^{ufd}-Ubc12^{core} with the E2-RING complexes structures suggest that there is overlap between the E1 and E3 binding sites on Ubc12 (Figure 5). Both NE1^{ufd} and the RING domains of c-Cbl and CNOT4 bind the same portions of the N-terminal helix of their respective E2 partners (Figure 5), which corresponds to roughly 10% of the NE1^{ufd} binding site on Ubc12^{core}. Indeed, UbcH5B's binding to CNOT4 is diminished upon mutation of UbcH5B's Lys4 and Lys8, which correspond to Ubc12's Leu32 and Lys36 in the NE1^{ufd} binding site (Winkler et al., 2004). Although Ubc12 is not known to interact with any HECT domain E3s, for comparison, the E6AP HECT domain binds the same ∼10% of the corresponding surface of UbcH7 (Huang et al., 1999). Moreover, UbcH7 makes extensive contacts with portions of c-Cbl outside the RING domain, corresponding to an additional ∼30% of the NE1^{ufd} binding site on Ubc12^{core} (Figure 5) (Zheng et al., 2000).

Multiple, different proteins binding to the same site on an E2 may affect the nature and processivity of ublp transfer cascades. If the E1 and E3 binding sites on an E2 overlap, then E1-catalyzed E2∼ublp thioester formation would require a free E2 as a substrate. Formation of polymeric ublp chains, or the extent of polymeric chain formation, may depend on the relative affinities of E1s and E3s for E2s in the free and the E2∼ublp thioester states. Indeed, the literature suggests that E1s and E3s display different affinities for different forms of their E2 partners. Loaded E1s bind their substrates, free E2s, prior to the transthiolation reaction (Hershko et al., 1983; Pickart and Rose, 1985). However, E1s readily release the product E2∼ubiquitin thioester complexes. By contrast, several studies suggest that E3s bind E2∼ubiquitin thioester complexes with high affinity and free E2s with low affinity. For example, data from Tanaka and colleagues suggest that SCF$^{\beta TRCP}$ binds the Ubc4∼ubiquitin thioester complex, but not free Ubc4 (Kawakami et al., 2001). Similarly, Siepmann and Haas found E3α to bind a Ubc2∼ubiquitin complex with nearly 10-fold higher affinity than free Ubc2 (Siepmann et al., 2003). E1 and E3 binding to a common site on E2 may serve

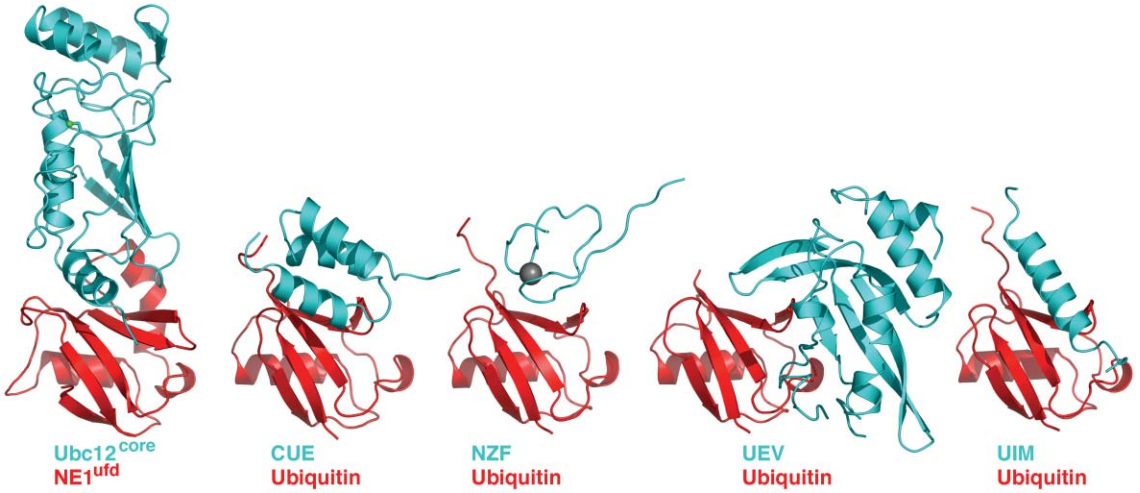

Figure 6. NE1^{ufd} Recruits Ubc12^{core} in a Manner Resembling Ubiquitin Interactions with Ubiquitin Binding Domains

Structures of NE1^{ufd}-Ubc12^{core}, ubiquitin in complex with the CUE domain from yeast Cue2 (Kang et al., 2003), ubiquitin in complex with the NZF domain of Npl4 (Alam et al., 2004), ubiquitin in complex with the UEV domain of TSG101 (Sundquist et al., 2004), and ubiquitin in complex with the UIM domain of Vps27 (Swanson et al., 2003) are shown from left to right, with ubiquitin in the same orientation as NE1^{ufd}. NE1^{ufd} and ubiquitin are shown in red, Ubc12^{core} and the ubiquitin binding domains are shown in cyan.

to order the sequence of reactions in the conjugation cascade and to ensure that the correct ublp is coordinated with the correct target.

Ubiquitin-Fold Domains: Common Protein-Protein Interaction Platforms in Ublp Transfer Cascades

The E1's ubiquitin-fold domain's interactions with Ubc12^{core} share common features with ubiquitin, ublp, and other ubiquitin-fold proteins' interactions with their partners. As examples, ubiquitin binds coupling of ubiquitin conjugation to ER degradation (CUE), Npl4 *Z*n finger (NZF), ubiquitin E2 *v*ariant (UEV), ubiquitin interacting motif (UIM), and other ubiquitin binding domains with its L8/I44/V70 hydrophobic patch exposed on its β sheet (Figure 6) (Alam et al., 2004; Kang et al., 2003; Prag et al., 2003; Sundquist et al., 2004; Swanson et al., 2003; Teo et al., 2004). The corresponding side chains in NE1^{ufd} all make critical interactions with Ubc12^{core}, although they are not hydrophobic.

One interaction between an ublp and its binding partner raises the intriguing possibility that some of the function of the E1's ubiquitin-fold domain may be related to structural mimicry of ubiquitin and ublps. The ublp SUMO interacts with its E2, Ubc9, in two ways (Johnson, 2004). Like other ublps, SUMO forms a covalent thioester complex with Ubc9 as an intermediate in the conjugation cascade. However, unlike other ublps, SUMO also forms a noncovalent complex with Ubc9. Although there is no structure for the noncovalent SUMO-Ubc9 complex, NMR and biochemical studies have revealed this interaction to involve SUMO's β sheet and Ubc9's N-terminal helix (Bencsath et al., 2002; Liu et al., 1999). Thus, noncovalent SUMO-Ubc9 may mimic the E1-E2 complex. Indeed, SUMO competes with E1 for binding to Ubc9, although the functional roles of the SUMO-Ubc9 interaction and the competition with E1 are not known (Bencsath et al., 2002).

In addition to the ubiquitin-fold domains of E1s, many other proteins in ubiquitin and ublp pathways adopt structures resembling ubiquitin. As examples, the Elongin B component of SOCS E3s resembles ubiquitin (Stebbins et al., 1999), as do domains of the parkin E3 (Kitada et al., 1998) and the proteasome-associated protein Rad23 (Watkins et al., 1993). As the molecular mechanisms of ubiquitin-fold domain proteins are further unraveled, it will be interesting to see whether the ubiquitin-like structures are coincidental, or whether they reflect functional mimicry.

Experimental Procedures

Protein Preparation

Unlabeled and ^{32}P-labeled human NEDD8, phosphorylated at an N-terminal protein kinase A site, terminating at Gly76, and representing the active form of NEDD8, were described previously (Walden et al., 2003b). Human NEDD8 E1 (APPBP1-UBA3), the mutant NEDD8 E1 lacking its ubiquitin-fold domain (NEDD8 E1 Δufd or APPBP1-UBA3Δufd), Ubc12, Ubc12^{core}, and mutant forms of these proteins and complexes were generated as described previously (Huang et al., 2004; Walden et al., 2003b). NE1^{ufd} corresponds to human UBA3 residues 347–442 and was expressed as a GST-fusion from pGEX4T3 (GE Healthcare) in BL21Gold(DE3) cells (Stratagene). After initial glutathione affinity chromatography and thrombin cleavage, the isolated NE1^{ufd} was purified to homogeneity by anion exchange and gel filtration chromatography. NE1^{ufd} and Ubc12^{core} were concentrated to 20–50 mg/ml in 25 mM Tris-HCl, 150 mM NaCl, 5 mM DTT (pH 7.6), aliquotted, and stored at −80°C.

Ubc12~NEDD8 Thioester Assay

Reactions were performed in 10 μl in 50 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, 5 mM ATP, 1 mM DTT, 0.3 U/ml inorganic pyrophosphatase, 0.3 U/ml creatine phosphatase, 5 mM creatine phosphate, 2 mg/ml ovalbumin (pH 7.6), with 5 μM ^{32}P-NEDD8, at 18°C. Reactions were quenched with 2x SDS buffer. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), dried, and visualized by autoradiography. Comparison of APPBP1-UBA3 and APPBP1-UBA3Δufd activity in Figure 1 was performed for 30 s with 1 nM E1 and the indicated Ubc12^{core} concentrations (0–235 μM). The time course of ^{32}P-NEDD8 transfer from APPBP1-UBA3Δufd (1 nM) to Ubc12^{core} (235 μM) was stopped at 2,

10, 30, and 60 min. NE1^{ufd} inhibition of Ubc12~^{32}P-NEDD8 thioester formation was carried out for 1 min with 1 nM APPBP1-UBA3, 0.1 μM Ubc12, and 0–20 μM NE1^{ufd}. The effects of mutations were assayed for 2 and 10 min with 1 nM E1 and either 0.2 μM full-length Ubc12 or 2.5 μM Ubc12^{core}. The Ubc12~^{32}P-NEDD8 thioester complexes were verified by reduction with 100 mM DTT.

Nondenaturing Electrophoretic Mobility Shift Assay

NE1^{Ufd} and Ubc12^{core} were mixed on ice for 30 min in 25 mM Tris-HCl, 150 mM NaCl, 5% (v/v) glycerol, 5 mM DTT (pH 7.6), separated on a 4.5% polyacrylamide gel as described previously (Bencsath et al., 2002), and visualized by Coomassie blue staining.

Crystallization, Data Collection, and Structure Determination

NE1^{Ufd} and Ubc12^{core} were mixed at a 1.5:1 molar ratio, and crystals were grown at 4°C by microseeding using the hanging drop vapor diffusion method by mixing 1:1 with 12.5%–15.5% (v/v) PEG 3.5k, 0.2 M $(NH_4)_2SO_4$, 0.1 M Bis-Tris, and 5 mM DTT (pH 5.5). The crystals form in $P2_12_12_1$ with a = 40.6 Å, b = 61.5 Å, c = 125.9 Å, and one complex per asymmetric unit. Crystals were flash-frozen in 22% (v/v) PEG 3.5k, 0.2 M $(NH_4)_2SO_4$, 0.1 M Bis-Tris, 5 mM DTT, and 20% (v/v) ethylene glycol (pH 5.5), prior to data collection at the 8.3.1 beamline at the Advanced Light Source (ALS), the X25 and X12b beamlines at the National Synchrotron Light Source (NSLS), and the Structural Biology Center-Collaborative Access Team (SBC-CAT) beamline at the Advanced Photon Source (APS). Reflection data were processed using HKL2000 (Otwinowski and Minor, 1997).

Initial phases were obtained using MAD data from crystals of a selenomethionine (SeMet)-labeled complex. To improve our chances of obtaining a high-quality initial electron density map, we made nine mutants of NE1^{ufd}, each with a Leu, Ile, or Phe residue substituted with Met, because NE1^{ufd} contains only a single solvent-exposed Met that we thought may end up being disordered in the complex. This proved to be a fortuitous approach, although not for the reason we anticipated. Using SOLVE (Terwilliger and Berendzen, 1999), we readily identified all of the expected SeMet sites, other than an N-terminal SeMet in Ubc12^{core} that is present due to cloning, for SeMet-labeled Ubc12^{core} in complex with seven of the SeMet mutants of NE1^{ufd}. However, the electron density maps for the Ubc12^{core}-NE1^{ufd} Leu394SeMet mutant complex were of substantially better quality than any of the other maps and were used for all further refinement. These maps revealed continuous, high-quality density for the entire ubiquitin-fold domain from NEDD8's E1, and allowed correction of misaligned sequence for the two C-terminal strands in a prior structure (Walden et al., 2003b). In the structures of NEDD8's E1, weak discontinuous density precluded identification of some loops and side chains in this domain, so parts of all previous structures of this domain were built as polyalanine (Huang et al., 2004; Walden et al., 2003a, 2003b). Therefore, the NE1^{ufd} structure reported here supercedes the previous structures of the ubiquitin-fold domain in NEDD8's E1 (Huang et al., 2004; Walden et al., 2003a, 2003b). The model was built manually in O (Jones et al., 1991) and refined to 2.4 Å resolution with CNS (Brünger et al., 1998) against data from the peak wavelength, with a final R factor of 24.2 and R_{free} of 25.9. The final model contains NE1^{ufd} (Leu394SeMet) residues 349–440, and Ubc12^{core} residues 27–183 and Gly-Ser-Met at the N terminus due to cloning. The fraction of residues in the most favored and additionally allowed regions of the Ramachandran plot are 90% and 9.5%, respectively, with no residues in a disallowed region. Details of refinement are listed in Table 1.

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Accession Numbers

The $NE1^{ufd}$-$Ubc12^{core}$ structure has been deposited in the RCSB Protein Data Bank under accession code 1Y8X.