E1-E2 Interactions in Ubiquitin and Nedd8 Ligation Pathways

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Background: Ubiquitin carrier protein (E2) recognition by ubiquitin activating enzyme (E1) defines fidelity in subsequent conjugation reactions.

Results: E2 transthiolation kinetics identify structural features defining the specificity of E1-E2 binding.

Conclusion: E2 paralogs contain a conserved E1 binding motif, and the E1 β-grasp domain is a specificity filter for E2 binding.

Significance: This defines structural features determining ubiquitin conjugation fidelity.

Initial rates of E1-catalyzed E2 transthiolation have been used as a reporter function to probe the mechanism of [125I]-ubiquitin transfer between activation and ligation half-reactions of ubiquitin conjugation. A functional survey of 11 representative human E2 paralogs reveals similar $K_m$ for binding to human Uba1 ternary complex ($K_{m(ave)} = 121 \pm 72$ nM) and $k_{cat}$ for ubiquitin transfer ($k_{cat(ave)} = 4.0 \pm 1.2$ s$^{-1}$), suggesting that they possess a conserved binding site and transition state geometry and that they compete for charging through differences in intracellular concentration. Sequence analysis and mutagenesis localize this binding motif to three basic residues within Helix 1 of the E2 core domain, confirmed by transthiolation kinetics. Partial conservation of the motif among E2 paralogs not recognized by Uba1 suggests that another factor(s) account for the absolute specificity of cognate E2 binding. Truncation of the paralogous domain from the Nedd8 activating enzyme has negligible effect on cognate Ubc12 transthiolation but abrogates E2 specificity toward non-cognate carrier proteins. Exchange of the β-grasp domains between ubiquitin and Nedd8 activating enzymes fails to reverse the effect of truncation. Thus, the conserved Helix 1 binding motif and the β-grasp domain direct general E2 binding, whereas the latter additionally serves as a specificity filter to exclude charging of non-cognate E2 paralogs in order to maintain the fidelity of downstream signaling.

Ligation of Class I ubiquitin-like proteins to cellular targets provides a fundamental and highly conserved strategy for regulating diverse processes within eukaryotes (1–4). These parallel but distinct conjugation pathways share a common, evolutionarily conserved mechanism initiated by activating enzymes (E1)$^5$ specific for each ubiquitin-like protein. The E1 paralogs couple ATP hydrolysis to formation of ternary complexes comprising a high energy thiolester-linked intermediate between the carboxyl terminus of the Ubl protein and an absolutely conserved cysteine of the enzyme in addition to a tightly bound adenylate intermediate that serves as the immediate precursor for the thiolester (5, 6). Although the E1 paralogs share marked homology in their primary amino acid sequences and catalyze parallel chemistries for their respective activation reactions, they exhibit absolute specificity for their cognate Ubl proteins. The activation mechanism and binding interactions defining cognate Ubl protein specificity have been partially revealed by kinetic, mutational, and structural analyses (5, 6).

The E1 paralogs also catalyze transfer of the internal ubiquitin-like protein thiolester to the active site cysteine of their cognate E2 (Ubc) carrier proteins (5–7). Eukaryotes express a superfamily of ubiquitin-specific E2 paralogs typically of 14–35-kDa molecular mass that support E3- and phenotype-specific functions (8, 9); in contrast, the Nedd8, SUMO, and ISG15 ligation pathways are specific for single E2 isoforms (10–12). The fidelity with which the E1 paralogs distinguish their cognate E2 isoform(s) is critical in precluding cross-talk among these

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5 The abbreviations used are: E1, generic term for activating enzymes of Class 1 ubiquitin-like proteins; E2/Ubc, ubiquitin carrier protein or ubiquitin-conjugating enzyme; E3, ubiquitin-protein isopeptide ligase; AppBp1-Uba3, heterodimeric Nedd8 activating enzyme (NAE1-UBE1C); Ubc2b, “b” isoform of the human/rabbit ortholog of S. cerevisiae Rad6/Ubc2 (also termed E2$_{rad6}$); Uba1, ubiquitin activating enzyme; SUMO, small ubiquitin-like modifier.
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parallel regulatory pathways. Because the E2 superfamily is characterized by marked conservation in sequence and structure within a shared ~150-residue core catalytic domain (8), fundamental questions remain regarding interaction surfaces through which E1 binds E2 and the determinates that allow E1 paralogs to distinguish cognate from non-cognate E2 family members.

Early work suggested that Uba1 engages the ubiquitin-specific Ubc2/Rad6 of Arabidopsis thaliana through its amino-terminal α-helix (13, 14), although yeast genetics also implicated binding at the carboxyl-terminal α-helix (15). Qualitative functional data from point mutants identified the paralogous amino-terminal α-helix of human Ubc9 in binding to the SUMO-specific Sae1-Sae2 heterodimeric activating enzyme (16). Structural studies suggest that the 26-residue amino-terminal extension peptide on Ubc12 contributes significantly to binding the Nedd8-specific AppBp1-Uba3 heterodimeric activating enzyme; however, this interaction is likely to be specific to the Nedd8 conjugation pathway because this peptide is unique to Ubc12 (17). Structural studies in which the amino-terminal extension is truncated from Ubc12 suggests that the paralogous amino-terminal α-helix of the catalytic core interacts with the carboxyl-terminal β-grasp fold of Uba3 (18). More recently, dynamic NMR structures implicate the identical interaction surface on Ubc9 for binding to the Sae1-Sae2 SUMO activating enzyme (19).

We have previously developed biochemically defined kinetic assays to quantify unambiguously the binding of human Ubc2/Rad6 to Uba1 and of N-end rule substrates to human E3s (7, 20, 21). The present studies extend these methods to compare binding of selected human E2 paralogs to Uba1 and to localize precisely the binding surface on human Ubc2b that interacts with Uba1. These studies show that Uba1 binds its cognate E2 paralogs with remarkably similar affinities, suggesting a common Uba1 interaction motif. Truncation and mutagenesis studies have localized this Uba1 binding motif to three relatively conserved basic residues within the amino-terminal α-helix of the E2 core domain. Other studies show for the first time that the carboxyl-terminal β-grasp domain conserved among E1 paralogs functions as a specificity filter to exclude non-cognate E2 isoforms and differentially contributes to the net binding affinity for human ubiquitin versus Nedd8 activating enzymes.

MATERIALS AND METHODS

Bovine ubiquitin was purchased from Sigma and purified to apparent homogeneity (22, 23). Recombinant Nedd8 was that used previously (24). Carrier-free Na125I and [2,8-3H]ATP were purchased from PerkinElmer Life Sciences. Ubiquitin and Nedd8 were radioiodinated by the Chloramine-T method using carrier-free Na125I (24, 25). A pGEX3X-Uba1a construct encoding the nuclear form of human ubiquitin activating enzyme (Uba1; NP_003325) was a gift of Dr. Alan L. Schwartz (Washington University School of Medicine, St. Louis, MO) and was used to express the amino-terminal GST fusion of wild type ubiquitin activating enzyme (26).6 The bicistronic pGEX plasmids encoding either wild type human Nedd8 activating enzyme (AppBp1-Uba3) or Nedd8 activating enzyme lacking the carboxy-terminal 95 amino acids of Uba3 (AppBp1-Uba3-Stop348; here referred to as AppBp1-Uba3-Δ348) were as described previously (27) and were generous gifts of Dr. Brenda Schulman (St. Jude Children’s Research Hospital, Memphis, TN).

Generation and Purification of E2 Proteins—The complete coding sequences for human UbcH7 (UBE2L3), UbcH8 (UBE2L6), UbcH6 (UBE2E1), UbcE2E (UBE2E2), and UbcM2 (UBE2E3) were subcloned by PCR into the indicated restriction sites and pGEX plasmids summarized in supplemental Table 1. The indicated point mutants of Ubc2b were generated by overlap extension PCR using pGEX-HsUbc2b as a template (20, 28). The complete coding regions for all clones were sequenced to preclude cloning artifacts. Recombinant human Ubc2b (UBE2B), Ubc3(UBE2R1), Ubc5A (UBE2D1), Ubc5B (UBE2D2), Ubc5C (UBE2D3), Ubc8 (UBE2H), E2epf (UBE2S), and Ubc12 (UBE2M) were as described previously (7, 23, 24, 29, 30). Escherichia coli BL21(DE3) cells harboring the pGEX-E2 plasmids were induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 37 °C (7). The GST-E2 fusions were purified on glutathione-Sepharose, and then the GST moiety was removed by thrombin cleavage and the E2 proteins were further purified to apparent homogeneity by passage through glutathione-Sepharose and Mono Q fast protein liquid chromatography (7). The activities of the conjugating enzymes were quantitated by stoichiometric 125I-ubiquitin thiolester formation (7) and compared with total protein determined spectrophotometrically using their theoretical 280 nm extinction coefficients. The apparently homogenous recombinant E2 paralogs exhibited different but reproducible yields and percentage of active protein: UbcH7 (10 mg/liter of culture; 20% active), UbcH8 (4 mg/liter of culture; 3% active), UbcH6 (20 mg/liter of culture; 30–40% active), UbcE2E2 (40 mg/liter of culture; 8% active), and UbcM2 (80 mg/liter of culture; 2% active). All purified E2 proteins were stored at −80 °C in aliquots to avoid progressive inactivation from repeated freeze-thaw cycles.

Generation of GST-Uba1Δ946—The amino-terminal GST fusion of wild type Uba1 human ubiquitin activating enzyme was expressed from pGEX3X-Uba1a (26). Deletion of the 118-residue carboxyl-terminal β-grasp domain of Uba1 paralogous to the carboxyl-terminal 95-amino acid region of Uba3 (supplemental Fig. 1) was achieved by PCR using Pfu polymerase. The amino-terminal 946-amino acid coding region of Uba1 was amplified using primers for the region of the gene bound by BamH1/Smal restriction sites. Following PCR amplification of the truncated gene (codons 1–946), the amplified DNA was subsequently cleaved with BamH1/Smal restriction enzymes, agarose gel-purified, and ligated into similarly restricted pGEX-4T1 to yield pGEX-4T1-Uba1Δ946. Fidelity of the truncation was confirmed by sequencing the complete cod-

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6 We do not refer to the GST moiety because it is present on all E1 paralogs and mutants used in these studies, unless specifically noted. Previous kinetic work demonstrates the presence of the GST moiety does not affect the enzymatic activity of human Uba1 (7).
ing region in the Protein and Nucleic Acid Core Facility at the Medical College of Wisconsin.

Generation of GST-Chimeric Uba1—To clone the 95-amino acid β-grasp domain of Uba3 into the paralogous position of Uba1, the coding region for the carboxyl-terminal 95 amino acids of Uba3 was amplified using primers for the region of the gene bound by Smal restriction enzymes. The amplified DNA was subcloned into pGEM-T. The subcloned DNA encoding the 95-residue β-grasp domain of Uba3 and the pGEX-4T1-Uba1Δ946 construct were digested with the Smal restriction enzyme, agarose gel-purified, and ligated. The correct orientation of the 95-amino acid region of Uba3 and the nucleotide sequence of the construct were confirmed by automated sequencing in the Protein and Nucleic Acid Core Facility at the Medical College of Wisconsin.

Generation of GST-Chimeric AppBp1-Uba3—The β-grasp domain of Uba3 was replaced by the paralogous β-grasp domain of Uba1 by introducing the carboxyl-terminal 118-amino acid coding region of the latter by overlap extension PCR (28). In the first round of PCR, the coding region for truncated Uba3A348 (codons 1–348) was amplified with a forward primer containing a HindIII restriction site and a reverse primer containing the 24 bases of Uba3 up to codon 348 fused to 21 bases, which corresponded to codons 946–952 of Uba1, residing immediately amino-terminal to the β-grasp domain of the latter. The carboxyl-terminal 118-codon fragment of Uba1 was amplified with a forward primer containing 24 bases of Uba3 up to codon 348 fused to 21 bases (codons 946–952) of the Uba1 β-grasp domain and a reverse primer containing the last 18 bases (codons 1053–1058) of Uba1 fused to a STOP codon, followed by a NotI restriction site. In the second round of PCR, the gel-purified PCR products of the first round were mixed and used as a template for amplification using primers flanking the HindIII/NotI restriction sites. After gel purification and HindIII/NotI digestion, the second round PCR product was ligated into the similarly digested pGEX-AppBp1-Uba3 vector. The coding sequence of the chimeric AppBp1-Uba3 construct was verified by direct sequencing of the complete coding region for Uba3.

Expression and Purification of GST-E1 Isoforms—E. coli BL21 (DE3) cells were transformed with pGEX vectors encoding either wild type or mutant forms of ubiquitin activating enzyme or Nedd8 activating enzyme. Recombinant proteins were expressed and purified as described previously (21). The purified proteins were analyzed by 7.5% SDS-PAGE followed by Western blotting and subsequent visualization using anti-GST antibodies and ECL detection. Protein concentrations for full-length wild type and mutant enzymes were normalized to GST content using quantitative Western blotting.

Enzyme Assays—Concentrations of active wild type, truncated, and chimeric Uba1 and AppBp1-Uba3 were determined by the stoichiometric formation of thiolester with free 125I-ubiquitin (~5000 cpm/pmol) or 125I-Nedd8 (~4000 cpm/pmol), respectively. Enzyme-bound ubiquitin [3H]adenylate formation was determined using [2,8-3H]ATP (3.6 × 10⁴ cpm/pmol) and measured by TCA-precipitable radioactivity (31, 32). Initial rates of E2-125I-ubiquitin and E2-125I-Nedd8 thiolester formation were measured by non-reducing SDS-PAGE in E1-catalyzed transthiolation assays at the indicated concentrations of ATP, 125I-ubiquitin or 125I-Nedd8, and recombinant human E2 (7). The binding affinities of ATP and ubiquitin for Uba1a and of Nedd8 for AppBp1-Uba3 were determined by transthiolation kinetics (7, 21). In the transthiolation kinetic assays, the binding affinities between E1 ternary complex and E2 isozymes were quantitated as a function of varying E2 concentration. The initial rates (vₒ) for transfer of activated ubiquitinin from E1 ternary complex to E2 were measured under valid conditions as required by Michaelis-Menten kinetics in which [E2]ₒ >> [E1]ₒ. In this assay, the initial net forward rate of the activation reaction (i.e. steady-state formation of E1 ternary complex) is measured by coupling the activation of ubiquitin or Nedd8 to the transthiolation of E2 under E1-limiting conditions. The initial velocities for E2 thiolester formation were calculated and plotted as initial velocities versus initial substrate (E2) concentrations. The dependence of vₒ on [E2]ₒ showed hyperbolic kinetics from which Kₘ and Vₘₐₓ values were calculated by non-linear regression analysis using GraFit. The corresponding kₘₐₓ was calculated from Vₘₐₓ/[E1]ₒ.

RESULTS

Human E2 Paralogs Bind Uba1 through Conserved Motif—The ubiquitin-specific E2 carrier proteins segregate into at least 15 discrete families having distinct phenotypes, of which at least three families (UbcH6, UbcH7, and UbcH8) are present only in higher eukaryotes, indicating that divergence and protein speciation have continued to adapt ubiquitin-dependent regulation to new roles as organisms have increased in complexity (33, 34). We were initially interested in understanding how members of the different E2 families compete for charging by Uba1. To address this question, we exploited E2 transthiolation as a facile reporter reaction for monitoring functional binding of representative human E2 paralogs to Uba1 ternary complex. Initial rates of E2-125I-ubiquitin thiolester formation were measured in order to determine values of Kₘ and kₘₐₓ as described previously (7), the results of which are summarized in Fig. 1A and supplemental Table 2. Each E2 isoform exhibited hyperbolic kinetics for vₒ versus [E2]ₒ below 1 μM, from which values of Kₘ (representing an intrinsic Kₐ) and kₘₐₓ (defined as Vₘₐₓ/[Uba1]ₒ) could be determined by nonlinear regression analysis (7). Above 1 μM, each of the E2 isoforms exhibited substrate inhibition similar to that previously found for human Ubc2b (7) and Ubc5B (35), indicating that this behavior is an intrinsic feature of Uba1-E2 interactions. We have previously demonstrated that the presence of the GST moiety on Uba1 has no effect on the kinetics of Uba1-catalyzed Ubc2b transthiolation (7, 21). The excellent agreement between the values for Kₘ and kₘₐₓ for Ubc2b and Ubc5B found here with GST-Uba1 (Fig. 1A and supplemental Table 2) versus those reported earlier with wild type human erythrocyte Uba1 (7, 35) further confirm this conclusion and validate the reproducibility of our experimental approach. Each of the E2 isoforms exhibited remarkably similar affinities for binding to Uba1 that ranged around an average value of 121 ± 72 nM (Fig. 1A and supplemental Table 2). The three Ubc5 isoforms consistently exhibited lower Kₘ values than the other E2 families, correlating with their qualitatively lower concentrations relative to Ubc2 in cultured human cell lines.
lines (7). Human Ubc3, UbcE2E2, and UbcM2 exhibited slightly higher values of $K_m$ (Fig. 1A). The latter two E2 paralogs and UbcH6 form a cluster of distinct E2 families with nearly identical E2 catalytic domains but distinguished by their amino-terminal extensions (supplemental Fig. 2). The various E2 paralogs also exhibited nearly identical $k_{cat}$ values of $4.0 \pm 1.2 \text{s}^{-1}$ (Fig. 1A and supplemental Table 2), presumably reflecting a common transition state geometry for ubiquitin transfer from the Uba1 ternary complex to bound E2 within the corresponding Michaelis complex. The similar binding affinities, measured as $K_m$ (7), for interaction of the uncharged E2 isoforms with Uba1 ternary complex suggest that the different ubiquitin-specific paralogs possess a conserved E1 binding surface, which we probed further using Ubc2b as a representative of the superfamily. Earlier studies identified E1 interaction sites within the amino-terminal helix (Helix 1) of Ubc12 within the Nedd8 pathway (18) and Ubc9 within the SUMO pathway (16), suggesting that Uba1 might also bind its cognate E2 paralogs through Helix 1. Sequence analysis of the amino-terminal $\alpha$ helix of selected human E2 isoforms identified five residues that were highly conserved among the E2 paralogs (Fig. 1B). Kinetic analysis of single alanine point mutants at each of the five conserved positions identified three residues within Helix 1 (positions 7, 8, and 11) for which $K_m$ was statistically greater than that of wild type protein (Fig. 1C and supplemental Table 3), indicating that these residues contribute to binding of Ubc2b to Uba1. In contrast, mutation of the conserved aspartate residues at positions 12 and 19 had no significant effect on the kinetics of E2 transthiolation (Fig. 1C), precluding their contribution in Ubc2b binding to the Uba1 ternary complex.

The conserved E1 binding motif comprises three basic residues within Helix 1 that correspond to (K/R)RXX(K/R). The three basic residues assume a triskelion of positive charges at one end of the overall structure (Fig. 1D). The E1 binding motif
resides on the same surface as the Loop 1/2 residues at positions 65 and 99 (Ubc2b numbering) previously identified as important for binding of other E2 paralogs to Ring and Hect domain ligases (36, 37) (Fig. 1, B and D). Mutation of these Loop 1/2 residues individually to alanine resulted in a significant increase in the affinity for Ubc2bN65A binding to Uba1 but a much smaller effect for Ubc2b T99A (Fig. 1C). Because mutation of Asn 65 affects Ubc2b binding, this position must also reside on the binding surface between Ubc2b and Uba1. Because the three basic residues are strongly conserved among E2 paralogs, this motif probably constitutes a common binding interface for all of the E1 paralogs. In contrast, none of the alanine point mutants that significantly increased $K_m$ exhibited a marked effect on $k_{cat}$ for Ubc2b transthiolesterification (Fig. 1C and supplemental Table 3); therefore, these residues must function exclusively in binding of Ubc2b to Uba1 ternary complex.

That Uba1 binds at the surface identified in Fig. 1D was also shown by the dramatic effect of the amino-terminal domain of GST-Ubc2b on $K_m$ and $k_{cat}$ for Uba1-catalyzed transthiolesterification of the fusion protein versus processed Ubc2b generated in situ immediately prior to the assay (Fig. 2). Both free Ubc2b and its GST precursor form identical amounts of $^{125}$I-ubiquitin thiolester in the standard end point assay used to quantitate E1 activity (supplemental Table 3); therefore, these residues must function exclusively in binding of Ubc2b to Uba1 ternary complex. The significantly ablated activity for the fusion protein cannot be caused by sequestration of GST-Ubc2b by dimerization with the GST moiety of GST-Uba1 because the former is in great excess in the kinetic assay in order to satisfy valid initial velocity conditions. The data are consistent with the amino-terminal GST moiety sterically hindering correct binding of Ubc2b to Uba1. Indeed, the affinity of GST-Ubc2b for Uba1 is sufficiently compromised that the rate data show no onset of saturation over a concentration range in which free Ubc2b readily reaches saturation (Fig. 2B). In addition, both sets of data were plotted on the same scale, rates for GST-Ubc2b would appear as if the E2 were inactive compared with the parallel processed Ubc2b. These observations emphasize that end point thiolester formation alone cannot be used as a valid indicator of catalytic competence because activity can be suppressed many orders of magnitude yet still show stoichiometric thiolester formation in the end point assay.

**Carboxyl-terminal β-Grasp Domain of Uba1 Contributes to Ubc2b Binding**

—Binding of Ubc12 to human AppBp1-Uba3 is suggested to require interaction of the E2 with the carboxy-terminal β-grasp domain of Uba3 (18), and analogous interactions have been proposed for Ubc9 binding to the β-grasp fold of the Sae1-Sae2 SUMO activating enzyme (19). In order to test the effect of this domain on Ubc2b transthiolesterification, the carboxy-terminal β-grasp fold of Uba1 corresponding to the carboxy-terminal 118 amino acids of the activating enzyme (supplemental Fig. 1) were deleted to yield Uba1Δ946. The Uba1 truncation retained the ability to form a $^{125}$I-ubiquitin thiolester intermediate with a ternary complex stoichiometry identical to that of wild type Uba1 (Table 1, Experiment 1). However, incubation of 20 nM Uba1Δ946 with 10 µM Ubc2b in the presence of saturating ATP and $^{125}$I-ubiquitin under initial velocity conditions resulted in a significantly slower rate of Ubc2b-$^{125}$I-ubiquitin thiolester formation (Fig. 3, lanes 3–9) compared with that found after 1 min for an equivalent concentration of wild type Uba1 and 100-fold less Ubc2b (Fig. 3, lane 2). The increase in the autoradiographic intensity of the Uba1-$^{125}$I-ubiquitin band beyond that for stoichiometric thiolester formation during the
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TABLE 1
Stoichiometry of Uba1 ternary complexes

|                          | 125I-Ubiquitin thiolester pmol | Ubiquitin [3H]adenylate pmol |
|--------------------------|--------------------------------|-----------------------------|
| **Experiment 1**         |                                 |                             |
| Uba1                     | 0.72 ± 0.02                     | 0.63 ± 0.04                 |
| Uba1Δ946                 | 0.67 ± 0.02                     | 0.60 ± 0.06                 |
| **Experiment 2**         |                                 |                             |
| Uba1                     | 0.31 ± 0.03                     | 0.29 ± 0.05                 |
| ChimericUba1             | 0.27 ± 0.02                     | 0.26 ± 0.02                 |

FIGURE 3. Deletion of the Uba1 β-grasp domain ablates the rate of Ubc2b transthiolation. Formation of Ubc2b-125I-ubiquitin thiolester catalyzed by Uba1 after a 1-min incubation (lane 2) or by Uba1Δ946 for the indicated times (lanes 4–9) was determined from incubations conducted in 50 mM Tris-HCl (pH 7.5) containing 2 mM ATP, 10 mM MgCl_2, 1 mM DTT, 1 mg/ml carrier BSA, 5 μM 125I-ubiquitin, 20 nM Uba1 or Uba1Δ946, and the indicated concentrations of Ubc2b at 37 °C. Aliquots of 25 μl were taken at the indicated times and quenched with an equal volume of SDS sample buffer without β-mercaptoethanol and then resolved by non-reducing 12% SDS-PAGE and visualized by autoradiography.

first minute represents a slow rate of Uba1 autoubiquitination noted previously (38), revealed by residual signal following reducing SDS-PAGE (not shown). In contrast, the bands in Fig. 3 associated with Ubc2b were labile under reducing conditions (not shown), confirming that the adducts are 125I-ubiquitin thiolestes (31, 39).

Initial rates of Ubc2b-125I-ubiquitin thiolester formation catalyzed by Uba1Δ946 showed hyperbolic kinetics with respect to Ubc2b, confirmed by the linearity of the corresponding reciprocal plots (7), and yielded a $K_m$ of 3.1 ± 1.7 μM for Ubc2b that represented a 30-fold lower affinity than found with wild type Uba1 (Table 2). The corresponding $\Delta G^*$ indicates that the β-grasp domain of Uba1 contributes approximately half of the overall Ubc2b binding energy of 9.7 kcal/mol. Thus, truncation of the β-grasp domain substantially reduces, but does not abrogate, interaction between Ubc2b and Uba1 ternary complex. However, the tripartite binding motif within Helix 1 of Ubc2b does not interact with the carboxy-terminal β-grasp domain because each of the alanine point mutants tested in Fig. 1C also exhibited an increased $K_m$ for binding to Uba1Δ946 ternary complex (Fig. 4), although the absolute magnitude of the effect for each of the point mutants was attenuated compared with that for wild type Uba1 (Fig. 1C). The decrease in magnitude for binding of the point mutants probably reflects the non-additivity observed in such experiments due to entropic considerations of quasi-independent but physically linked binding sites (40).

Unexpectedly, the $k_{cat}$ for Uba1Δ946-catalyzed transthiolation of Ubc2b was 3.5 × 10^4-fold less than that of wild type Uba1, representing a 10^6-fold decrease in catalytic efficiency ($k_{cat}/K_m$). Although truncating the β-grasp domain of Uba1 effectively inactivates the enzyme, loss of the domain had no effect on ATP or ubiquitin binding determined from their respective concentration dependences (7) (Table 3). Therefore, the significant reduction in $k_{cat}$ requires that the carboxyl-terminal domain contribute to transition state stabilization during the transthiolation reaction ($\Delta G^* = 4.7$ kcal/mol).

Stoichiometry and Kinetics of Chimeric Uba1—Because potential catalytic groups on the β-grasp domain are too far removed directly to participate in the reaction (6), the marked effect of Uba1Δ942 on $k_{cat}$ requires that the domain participates in optimally positioning Ubc2b for nucleophilic attack by Cys^88 on the Uba1-ubiquitin thiolester. To examine this, we exchanged the paralogous β-grasp domains of Uba3 and Uba1 as detailed under “Materials and Methods” to yield chimeric Uba1 and chimeric AppBp1-Uba3. Identical ternary complex stoichiometries for wild type and chimeric Uba1 (Table 1, Experiment 2) were consistent with earlier observations that the β-grasp domain was not required in the Uba1 catalytic cycle (Table 1, Experiment 1). Other rate studies with chimeric Uba1 yielded $K_m$ values for ATP and ubiquitin that were in good agreement with those of wild type enzyme (Table 3). Kinetic studies of chimeric Uba1-catalyzed transthiolation with respect to Ubc2b yielded values of $k_{cat}$ and $K_m$ that were similar to those of Uba1Δ946 (Table 2), thus precluding a passive steric role for the β-grasp domain. Unfortunately, the extreme instability of the corresponding chimeric AppBp1-Uba3 protein prevented functional characterization of this mutant.

Because the β-grasp domain is thought to interact directly with E2 based on qualitative “pull down” observations with AppBp1-Uba3 (18), it presumably contributes to cognate E2 specificity through such binding interactions. To test this, we examined the ability of chimeric Uba1 to catalyze Ubc12-125I-ubiquitin thiolester formation. Although incubating 0.3 pmol of AppBp1-Uba3 with 1 pmol of Ubc12 resulted in stoichiometric Ubc12-125I-Nedd8 thiolester formation within 1 min (Fig. 5, lane 2), incubation of 1 pmol of wild type Uba1 with 10 pmol of Ubc12 showed no detectable transthiolation within 5 min at

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7 Because binding energies are not additive, due to the difficulty in accounting for the entropic contribution of connectivity between different binding sites, the 50% contribution is only approximate.
37 °C (Fig. 5, lane 5), confirming the absolute specificity of Uba1 in selecting against its non-cognate E2 isoforms (24). However, incubation of chimeric Uba1 with 10 pmol of Ubc12 under the same conditions as for wild type activating enzyme resulted in a significant but non-stoichiometric formation of Ubc12-125I-ubiquitin thiolester formation (Fig. 5, lane 4), consistent with a role for the β-grasp domain in defining cognate E2 specificity.

Subsequent kinetic characterization of chimeric Uba1-catalyzed 125I-ubiquitin transthiolation revealed hyperbolic kinetics with respect to Ubc12 and yielded a $K_m$ of 0.72 ± 0.20 μM that approaches the value of 0.12 ± 0.04 μM for wild type recombinant AppBp1-Uba3 binding of Ubc12 (Table 2). These observations are consistent with the β-grasp domain contributing to Ubc12 binding and specificity, the latter property of

### TABLE 2

**Kinetic constants for Ubc2b and Ubc12 transthiolation**

The E2 concentration dependence on the rate of E1-catalyzed transthiolation was determined under initial velocity conditions as described under “Materials and Methods.” Incubations of 50 μl final volume contained 20 nm of the indicated E1, 2 mM ATP, and 5 μM 125I-ubiquitin or 125I-Nedd8 and were allowed to proceed for 20 min at 37 °C before quenching by the addition of 50 μl of SDS sample buffer. The resulting thiolestes were quantitated as described previously (21, 33).

| Uba1    | Ubc2b | $K_m$ (μM) | $k_{cat}$ (s⁻¹) | $k_{cat}/K_m$ (M⁻¹s⁻¹) |
|---------|-------|------------|-----------------|-------------------------|
|         |       |            |                 |                         |
| Uba1    |       | 0.11 ± 0.01| 3.4 ± 0.1       | 3.1 x 10⁷               |
| Uba1A946|       | 3.1 ± 1.7  | 10 ± 1.4 x 10⁵  | 32                      |
| chimeric Uba1 | | 2.6 ± 0.7  | 9.7 ± 0.8 x 10⁴ | 37                      |
| AppBp1-Uba3 | | -        | -               | -                       |
| AppBp1-Uba3-Δ348 | | -        | -               | -                       |

| Ubc12   | $K_m$ (μM) | $k_{cat}$ (s⁻¹) | $k_{cat}/K_m$ (M⁻¹s⁻¹) |
|---------|------------|-----------------|-------------------------|
|         |            |                 |                         |
|         |            | 0.84 ± 0.14     | 1.4 ± 0.1 x 10⁴         |
|         |            | 5.8 ± 0.4 x 10³ | 8.1 x 10³               |
|         |            | 3.9 ± 0.6       | 3.3 x 10⁷               |

37 °C (Fig. 5, lane 5), confirming the absolute specificity of Uba1 in selecting against its non-cognate E2 isoforms (24). However, incubation of chimeric Uba1 with 10 pmol of Ubc12 under the same conditions as for wild type activating enzyme resulted in a significant but non-stoichiometric formation of Ubc12-125I-ubiquitin thiolester formation (Fig. 5, lane 4), consistent with a role for the β-grasp domain in defining cognate E2 specificity.

**TABLE 3**

**Kinetic constants for Ubc2b transthiolation**

Transthiolation kinetic assays were performed identical to those of Table 2 with the exception that either [ATP]₀ or [125I-ubiquitin]₀ was varied at constant Ubc2b of 0.5 μM and 5 μM 125I-ubiquitin or 5 mM ATP, respectively.

| $K_m$ for ATP | $K_m$ for ubiquitin | $k_{cat}$ |
|---------------|--------------------|-----------|
| μM            | μM                 | s⁻¹       |
| Uba1          | 5.5 ± 0.7          | 0.8 ± 0.1 | 3.4 ± 0.1 |
| Uba1A946      | 5.4 ± 2.3          | 0.6 ± 0.2 | 13 ± 1.5 x 10⁻⁵ |
| Chimeric Uba1 | 4.5 ± 2.2          | 1.2 ± 0.3 | 7.5 ± 0.6 x 10⁻⁵ |

37 °C (Fig. 5, lane 5), confirming the absolute specificity of Uba1 in selecting against its non-cognate E2 isoforms (24). However, incubation of chimeric Uba1 with 10 pmol of Ubc12 under the same conditions as for wild type activating enzyme resulted in a significant but non-stoichiometric formation of Ubc12-125I-ubiquitin thiolester formation (Fig. 5, lane 4), consistent with a role for the β-grasp domain in defining cognate E2 specificity.

Subsequent kinetic characterization of chimeric Uba1-catalyzed 125I-ubiquitin transthiolation revealed hyperbolic kinetics with respect to Ubc12 and yielded a $K_m$ of 0.72 ± 0.20 μM that approaches the value of 0.12 ± 0.04 μM for wild type recombinant AppBp1-Uba3 binding of Ubc12 (Table 2). These observations are consistent with the β-grasp domain contributing to Ubc12 binding and specificity, the latter property of
A Conserved E2 Binding Site within E1

TABLE 4

| Kinetic constants for Ubc12 transthiolation |
|---------------------------------------------|
| Transthiolation kinetic assays were performed identical to those of Table 2 with the exception that either [ATP]_o or [125I-Nedd8]_o was varied at constant Ubc12 of 0.5 and 5 μM 125I-Nedd8 or 5 mM ATP, respectively. |
| K_m for ATP | K_m for Nedd8 | k_cat |
| μM | μM | s^{-1} |
| AppBp1-Uba3 | 103 ± 12 | 0.95 ± 0.18 | 3.5 ± 0.2 |
| AppBp1-Uba3Δ348 | 20 ± 1.9 | 0.4 ± 0.03 | 3.1 ± 0.3 |

which can be transferred to a non-cognate activating enzyme, such as Uba1.

The enhanced k_cat of 5.8 ± 0.4 × 10^{-3} s^{-1} for chimeric Uba1-catalyzed Ubc12 transthiolation compared with that for Ubc2b (Table 2) suggests that binding through the β-grasp domain also translates into enhanced catalytic specificity. However, because the k_cat for Ubc12 transthiolation catalyzed by chimeric Uba1 is considerably less than that of 3.9 ± 0.6 s^{-1} for wild type AppBp1-Uba3, other active site residue(s) within the AppBp1-Uba3 ternary complex must also be important for Ubc12 recognition and transthiolation. This conclusion is consistent with the structure for AppBp1-Uba3-Ubc12N26 peptide complex in which the amino-terminal 26-residue extension of Ubc12 is anchored in a groove generated by loops conserved in Uba3 but not in other activating enzymes (41) and a more complete structure of Ubc12-Nedd8 thiolester bound to the AppBp1-Uba3 ternary complex (42).

β-Grasp Domain Is a Specificity Filter for E2 Binding—Given the apparent role of the Uba3 β-grasp domain in defining binding and catalytic specificity for Ubc12 transthiolation, we quantitatively re-examined the effect of deleting the domain using the bicistronic vector encoding AppBp1-Uba3Δ348 (27). Deletion of the β-grasp domain from Uba3 had no effect on 125I-Nedd8 thiolester formation within the AppBp1-Uba3Δ348 ternary complex because the time courses for 125I-Nedd8 thiolester formation by wild type and truncated enzymes were qualitatively similar. Other kinetic studies demonstrated that wild type and truncated AppBp1-Uba3 had similar affinities for 125I-Nedd8 (Table 4). However, when ATP was varied at saturating 125I-Nedd8 (5 μM) and Ubc12 (0.5 μM), AppBp1-Uba3Δ348 consistently exhibited a 20-fold lower K_m for ATP than observed for wild type AppBp1-Uba3 (Table 4).

Although truncating the putative β-grasp domain of Uba1 decreased the affinity for Ubc2b binding ~30-fold, kinetic analysis showed that truncation of the paralogous domain from Uba3 had only a ~3-fold effect on binding of its cognate Ubc12 (Table 2), corresponding to a ΔΔG_binding of 0.6 kcal/mol compared with a ΔΔG_binding of 2.0 kcal/mol for Ubc2b binding to Uba1Δ946. Therefore, the β-grasp domain of AppBp1-Uba3 contributes significantly less to Ubc12 binding than Uba1 does to Ubc2b binding. More important, truncation of the β-grasp domain of Uba3 had relatively little effect on the k_cat for AppBp1-Uba3Δ348-catalyzed Ubc12 transthiolation compared with wild type enzyme, in contrast to the marked effect of the paralogous truncation on ubiquitin activating enzyme activity (Table 2). The latter observation indicates that the remaining interaction surface is sufficient to promote the transfer of activated Nedd8 from the active site cysteine of AppBp1-Uba3Δ348 to active site cysteine of Ubc12. This result is consistent with the observations with chimeric Uba1 because the kinetic constants for formation of Ubc12-ubiquitin and the Ubc2b-ubiquitin thiolesters by chimeric Uba1 suggest that there are additional residue(s) other than the β-grasp domain of Uba3 that are involved in cognate E2 binding and transthiolation.

Because the β-grasp domain of AppBp1-Uba3 contributes relatively little to the overall binding of Ubc12, it is difficult to reconcile the enhanced affinity of Ubc12 for chimeric Uba1 unless, counterintuitively, the β-grasp domain functions in defining specificity principally by excluding non-cognate E2 paralogs within the context of the wild type activating enzyme. That the β-grasp domain functions as a context-specific exclusionary specificity filter is demonstrated by our observation that Uba1Δ946 also catalyzes 125I-ubiquitin transthiolation to Ubc12 with kinetics similar to those of chimeric Uba1 (Table 2). Therefore, it is the absence of the wild type β-grasp domain and not the exchange with the paralogous Uba3 domain that enables chimeric Uba1 to catalyze Ubc12 transthiolation. As additional proof, wild type AppBp1-Uba3 is unable to catalyze 125I-Nedd8 transthiolation to Ubc2b (Fig. 6, lane 3), as we have shown previously (24); however, in a parallel incubation, AppBp1-Uba3Δ348 catalyzes a slow but detectable rate of 125I-Nedd8 transthiolation to Ubc2b (Fig. 6, lanes 5), as predicted by the specificity filter model. Unfortunately, the marked instability of AppBp1-Uba3Δ348 (t_{1/2} = 6 min at 37 °C) together with the significantly reduced rate evident in lane 5 versus lane 3 of Fig. 6 precludes detailed kinetic analysis. However, from the rate of Ubc2b-125I-Nedd8 thiolester formation in Fig. 6 (lane 5) and [AppBp1-Uba3Δ348], determined by end point 125I-Nedd8 thiolester formation (see “Materials and Methods”), we can estimate a k_cat/K_m of 273 M^{-1} s^{-1}, a value similar to that for
UbaΔ946-catalyzed formation of Ubc12-125I-ubiquitin formation of 167 M⁻¹ s⁻¹ (Table 2). Correspondence in the $k_{\text{cat}}/K_m$ values for the two truncated enzymes catalyzing non-cognate transthiolation reactions is consistent with a basal E1-catalyzed transthiolation rate for nonspecific E2 charging.

**DISCUSSION**

The E1 enzymes catalyze the essential first step of ATP-coupled activation that poises these pathways for subsequent conjugation of their cognate ubiquitin-like proteins to specific cellular targets. Marked sequence homology among the E1 paralogs anticipates a conserved mechanism for these enzymes, previously demonstrated empirically (24, 43, 44). The E1 enzymes also serve critical functions in defining the absolute specificity with which they discriminate among the ubiquitin-like proteins and the E2 acceptors of the activated polypeptides (6). The present work has exploited transthiolation kinetics as a reporter function to probe for the first time the specificity of the ubiquitin activating enzyme for its cognate E2 isoforms.

Marked conservation of the core catalytic domain among members of the E2 superfamily suggests that the intramolecular transthiolation reaction between E1 ternary complex and the active site cysteine of the bound E2 share a common chemical mechanism (5, 6, 33). The present observation that different E2 families exhibit remarkably similar $K_m$ and $k_{\text{cat}}$ values for Uba1-catalyzed transthiolation represents the first empirical evidence supporting a common interaction motif for E2 binding and shared transition state for transthiolation (Fig. 1A and supplemental Table 2). The excellent correspondence among $K_m$ and $k_{\text{cat}}$ values for E2 transthiolation reported here for ubiquitin and those reported previously for Nedd8 (24) and SUMO (6). The present work has exploited transthiolation kinetics as a reporter function to probe for the first time the specificity of the ubiquitin activating enzyme for its cognate E2 isoforms.

Sequence analysis localizes this motif to Helix 1 of the core catalytic domain, and the consequence of alanine-scanning mutagenesis for the kinetics for transthiolation identifies three basic residues as the Uba1 binding motif (Fig. 1 and supplemental Table 3). Of the three residues, Arg7 (Ubc2b numbering) contributes most significantly to Uba1 binding affinity (Fig. 1C); interestingly, this residue is not conserved in the E2 paralogs for Nedd8 (Ubc12), SUMO (Ubc9), and ISG15 (UbcH8) (Fig. 1B), suggesting that it may in part contribute to the ability of E1 paralogs to distinguish cognate from non-cognate E2 species. Otherwise, features of this recognition mechanism appear to be generally applicable to other members of the E1 family because the E1-E2 binding motif identified in Fig. 1 is relatively conserved within the ubiquitin-like protein ligation pathways.

Other features of the respective E1-E2 interactions must define critical aspects of pathway specificity. The motif present in Helix 1 is clearly not the only E1-E2 binding interface, although it quantitatively appears to account for at least 40% ($\Delta\Delta G_{\text{binding}} = 3.9$ kcal/mol) of the wild type binding energy. The predicted $K_m$ in the absence of the motif would be $\sim 70 \mu M$ compared with 100 nM for wild type Ubc2b.⁷ We note that recent work has shown that the position corresponding to Ser120 of Ubc2 also interacts with Uba1 and defines the quantitative difference in affinity between Ubc2 and Ubc5 paralogs as well as regulated changes in function and affinity upon phosphorylation (35).

These observations suggest that members of the ubiquitin-specific E2 superfamily compete for charging by Uba1 largely as a function of differences in their intracellular concentrations rather than intrinsic differences in their affinities ($K_m$) or catalytic competencies ($k_{\text{cat}}$) with the activating enzyme. This conclusion differs from that predicted by earlier transthiolation data for which $K_m$ and $k_{\text{cat}}$ values span many orders of magnitude among a much smaller group of E2 paralogs tested and between identical isoforms examined here (45). The disparity between the two studies probably results from the intrinsic uncertainty in the earlier data for which E1 and E2 concentrations were determined by total protein (45) rather than by functional stoichiometric ubiquitin thiolester assays (current study).

Although all E2 isozymes share marked sequence homology and a conserved core structure, ubiquitin activating enzyme does not catalyze a transthiolation reaction with either Nedd8-specific (Ubc12) or SUMO-specific (Ubc9) E2 paralogs, even at high E2 concentrations (24, 45, 46). Detailed aspects of the E1-E2 interaction surface that confer absolute specificity for only the cognate E2 paralog(s) remain poorly understood. The first evidence for a specific interaction surface between Nedd8 activating enzyme and its cognate E2 was provided by the structure for AppBp1-Uba3 in complex with the 26-residue amino-terminal extension of Ubc12 (17). Huang et al. (17) demonstrated that the 26-residue segment binds to Nedd8 activating enzyme at a unique loop sequence within Uba3. Because the 26-residue amino-terminal extension of Ubc12 and the complementary groove present on Uba3 are unique to Nedd8 conjugation, other features must define E2 specificity for the remaining ubiquitin-like ligation pathways. To understand how Uba1 interacts with its cognate E2 isoforms, we used the structure of AppBp1-Uba3 as a model (27). Structural studies have previously revealed that the carboxyl-terminal $\sim 95$ residues of Uba3 adopt a $\beta$-grasp fold similar to ubiquitin, and qualitative gel shift assays suggest that truncation of the domain qualitatively abrogates Ubc12 binding (27). Sequence comparison of the $\beta$-grasp domain of Uba3 with the paralogous region within Uba1 revealed only 14% homology between the carboxyl-terminal $\sim 100$ residues of human Uba1 and Uba3 (supplemental Fig. 1). A comprehensive sequence analysis of proteins with known $\beta$-grasp folds has identified six hydrophobic residues constituting the hydrophobic core interface between the $\alpha$-helix and the $\beta$-sheet that are essential for the “ubiquitin fold” (47). When we re-examined the sequence alignment of the carboxyl-terminal $\sim 100$-residues of Uba1 and Uba3 paralogs, these six hydrophobic residues were absolutely conserved (supplemental Fig. 1), supporting the hypothesis that the carboxyl-terminal $\sim 100$ residues of ubiquitin activating enzyme adopt a $\beta$-grasp fold.

To quantitatively analyze the role(s) of the $\beta$-grasp domains of the Nedd8 and ubiquitin activating enzymes in binding their cognate E2 paralogs, we performed Ubc12 and Ubc2b transthiolation assays, respectively, on recombinant human Uba1 and AppBp1-Uba3 enzymes in which the $\beta$-grasp domains had been truncated. Truncation of the $\beta$-grasp domains from Uba1
or Uba3 had no effect on the stoichiometry of ternary complex formation (Table 1) and little or no effect on the binding affinities for the ATP and ubiquitin co-substrates (Tables 3 and 4). Therefore, the β-grasp domains are not required for the E1 catalytic cycle leading to the corresponding ternary complexes. These data also indicate that truncation of the carboxyl-terminal domain does not generate significant structural perturbations in the adenylate and the thiolester domains of the ubiquitin or Nedd8 activating enzymes.

Earlier studies by Huang et al. proposed that the β-grasp domain of human Uba3 was essential for Ubc12 binding and transthiolation because truncation blocked both processes (18). In contrast, the present studies show that truncation of the β-grasp domain from Uba3 had negligible effect on either the $K_m$ for Ubc12 binding or the $k_{cat}$ for Ubc12 transthiolation (Table 2), indicating that the domain is dispensable for Nedd8 transthiolation, a conclusion consistent with the absence of a paralogous β-grasp domain in wild type Saccharomyces cerevisiae Uba3. These observations suggest that other regions, presumably the unique 26-residue amino-terminal extension of Ubc12, provide compensatory contributions to Ubc12 binding and Nedd8 transthiolation by assuming the role of the β-grasp domain. In the earlier studies of Huang et al. (18), the effect of truncating the β-grasp domain on AppBp1-Uba3 ternary complex formation was not examined, and we believe that the apparent discrepancy results from the destabilizing effect of truncation on Uba3 activity observed in the present studies, reinforcing the importance of quantitative functional assays in assessing the effects of genetic manipulations. The relative absence of contributions from the β-grasp domain of Uba3 appears to be unique to the Nedd8 conjugation pathway because deletion of the paralogous β-grasp domain from Uba1 reduced Ubc2b binding by ~30-fold compared with wild type Uba1 (Fig. 2 and Table 2). The corresponding $\Delta G_{binding}$ value of 4.2 kcal/mol indicates that the β-grasp domain of Uba1 contributes approximately half of the Ubc2b binding energy of 9.7 kcal/mol; therefore, the domain is necessary but not sufficient for Ubc2b binding.

The 10-fold difference in relative contribution of the respective β-grasp folds for Ubc2b versus Ubc12 binding was surprising given the sequence and structural conservation in the components and the nearly identical $K_m$ values (24) but can potentially be reconciled by contributions from the unique amino-terminal extension on Ubc12. It appears that the amino-terminal extension has assumed much of the affinity and positional contributions of the E1β-grasp domain in the Nedd8 pathway, based on the results of its truncation (18). A more dramatic effect was observed on $k_{cat}$ for the Ubc2b transthiolation reaction, which decreased from 3.4 ± 0.1 to 10 ± 1.4 × 10⁻³ s⁻¹ with the truncation of Uba1 β-grasp domain (Table 2). The corresponding catalytic efficiency ($k_{cat}/K_m$) was reduced ~10⁶-fold compared with wild type enzyme, corresponding to a transition state destabilization (Δ$\Delta G^\#: 9.5$ kcal/mol, using the empirical $k_{cat}/K_m$ relationship identified by Wolfenden (48). The effect on $k_{cat}$ could arise from a purely steric role for the β-grasp domain of Uba1 in positioning the bound Ubc2b molecule for the optimum geometry of nucleophilic attack on the Uba1-ubiquitin thiolester bond. Alternatively, the $k_{cat}$ effect could be a consequence of ablated Ubc2b binding energy that would otherwise contribute to transition state stabilization via entropy-enthalpy compensation. In order to distinguish between these hypotheses and to test if the domain is involved in specific binding interactions between E1 and its cognate E2, we exchanged the paralogous β-grasp domains of the ubiquitin and Nedd8 activating enzymes. That similar binding affinities are observed between Ubc2b and either Uba1Δ946 or chimeric Uba1 indicates that the β-grasp domain of Nedd8 activating enzyme does not contribute to Ubc2b binding (Table 2). Furthermore, the nearly identical $k_{cat}$ values for Ubc2b thiolester formation with either the truncated or chimeric Uba1 mutants rule out a purely steric contribution of the β-grasp domain to transition state stabilization. Therefore, it is most likely that the $k_{cat}$ effect accompanying truncation reflects specific orientation of the bound E2 within the Michaelis complex contributed by interaction with the β-grasp domain.

Interestingly, the “domain swap” experiments reveal an unexpected role for the β-grasp domain in defining the specificity of E2 recognition. Wild type Uba1 does not catalyze detectable Ubc12 transthiolation (Fig. 5, lane 5) (24); however, both Uba1Δ946 and chimeric Uba1 catalyze Ubc12-155I-ubiquitin thiolester formation (Table 2 and Fig. 5, lane 5), indicating that loss of the Uba1 β-grasp domain and not the origin of this domain defines E2 specificity. Similarly, AppBp1-Uba3 does not catalyze detectable Ubc2b transthiolation (Fig. 6, lane 3) (24) unless the paralogous β-grasp domain is deleted (Fig. 6, lane 5). These results require that, independent of any contribution to E2 binding, the β-grasp domains define carrier protein specificity by excluding non-cognate paralogs. The importance of specificity by binding exclusion rather than affinity is being increasingly appreciated, most recently in accounting for the substrate specificity of caspase-3 versus caspase-9 (49) and editing/proofreading by aminoacyl-tRNA synthetases (50).

The present quantitative studies demonstrate that E2 binding to E1 ternary complex proceeds through a conserved tripartite interaction motif contained in Helix 1 of the conserved core domain that accounts for a significant fraction of overall E2 binding affinity. The presence of this conserved binding motif and the remarkably similar $K_m$ and $k_{cat}$ values exhibited by the various E2 paralogs suggest that they compete for Uba1-catalyzed charging through differences in their intracellular concentrations. For the ubiquitin-specific E2 paralogs, additional binding contributions arise from interaction with the carboxyl-terminal β-grasp domain, which is independent of the Helix 1 motif. Unexpectedly, the β-grasp domain also confers specificity for the cognate E2 by serving as a filter to exclude non-cognate paralogs, thus ensuring fidelity in downstream signaling.

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REFERENCES
1. Aguilar, R. C., and Wendland, B. (2003) Curr. Opin. Cell Biol. 15, 184 – 190
2. Weissman, A. M. (2001) Nat. Rev. Mol. Cell Biol. 2, 169 – 178
