Conservation in the Mechanism of Nedd8 Activation by the Human AppBp1-Uba3 Heterodimer*

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Human Nedd8-activating enzyme AppBp1-Uba3 was purified to apparent homogeneity from erythrocytes. In the presence of [2,8-3H]ATP and 125I-Nedd8, heterodimer rapidly forms a stable stoichiometric ternary complex composed of tightly bound Nedd8 [125I]adenylate and Uba3-125I-Nedd8 thiol ester. Iso enzyme exchange kinetics show that the heterodimer follows a pseudo-ordered mechanism with ATP the leading and Nedd8 the trailing substrate. Human AppBp1-Uba3 follows hyperbolic kinetics for HsUbc12 transthiolation with 125I-Nedd8 substrate. Human AppBp1-Uba3 catalyzed activation of ubiquitin and HsUbc12 transthiolation. However, modest inhibition of 125I-Nedd8 ternary complex formation by unlabeled ubiquitin suggests a Kd > 300 μM for ubiquitin. Alanine 72 of Nedd8 is a critical specificity determinant for AppBp1-Uba3 binding because 125I-UbR72L undergoes heterodimer-catalyzed hyperbolic HsUbc12 transthiolation and yields Kcat = 0 ± 0.3 s⁻¹. These observaions demonstrate remarkable conservation in the mechanism of AppBp1-Uba3 that mirrors its sequence conservation with the Uba1 ubiquitin-activating enzyme.

Class I ubiquitin-like proteins exert their biological effects through covalent conjugation to their respective target proteins via distinct ligation pathways that function in parallel to those of ubiquitination. The ubiquitin-like proteins include Ubx, Nedd8, Hub1, IG1, FAT10, and Apg12 among others, reviewed in Ref. 10. Generally, conjugation of ubiquitin-like proteins modulates the protein-ligand interactions of their target rather than committing the target protein to degradation, the role most associated with ubiquitin ligation. Among the ubiquitin-like proteins, Nedd8 is the most closely related to ubiquitin with 58% identity between human paralogs (10). Nedd8 and its plant ortholog Rub1 are conjugated to Cdc53/Cull1 (11, 12) and other members of the Cullin family of proteins (13). Cullins are essential structural subunits of the Skp1-based (SCF, Skp1, Cul1, F-box) and elongin B/C-based families of ubiquitin protein ligases (E3), reviewed in Refs. 14 and 15. Conjuction of Nedd8 to Cul1 and Cul2 requires the Ring finger protein Roc1/Rbx1/Hrt1, which serves as a docking adapter (16, 17). The attachment of Nedd8 to Cullin isoforms is not required for the intrinsic ubiquitin ligase activity of the SCF complex; however, it enhances ubiquitin chain formation through activation of the cognate E2 ubiquitin-conjugating enzyme (12, 18). Because of the central role of SCF and elongin B/C ubiquitin ligases in critical regulatory processes within eukaryotes, Nedd8 conjugation is an essential post-translational modification that is subject to considerable recent interest (12, 15).

The ATP-coupled activation of Nedd8 that is required for subsequent charging of the Nedd8-specific Ubc12-conjugating enzyme is catalyzed by heterodimeric AppBp1-Uba3 in humans (4, 19). Human Uba3 shows 43% homology to the carboxy-terminal 500 residues of the human ubiquitin-activating enzyme HsUba1 and encompasses the putative ubiquitin adenylate active site identified by homology to the MoeB subunit of molybdopterin synthase (20, 21). Deletion of Uba3 is embryonic lethal in mice, arising from a mitotic defect in the G1/G0 transition and the resulting accumulation of cyclin E and β-catenin (22, 23), both targets of SCF ligases (24). However, the Uba3-catalyzed activation of Nedd8 exhibits an absolute requirement for AppBp1, a protein first identified by its interaction with the carboxyl terminus of amyloid precursor protein and that has marked homology to the amino-terminal half of Uba1 (20, 21, 25). Overexpression of AppBp1 rescues the temperature-sensitive ts41 mutation of Chinese hamster lung cells by driving S-M checkpoint progression through Nedd8 conjugation (26). The recent 2.6 Å structure of human AppBp1-Uba3 confirms that AppBp1 is required in part to contribute a short conserved active site segment first identified in the mechanistically related MoeB subunit of molybdopterin synthase (21, 27).

In the activation of ubiquitin, Uba1 forms a ternary complex composed of 1 eq each of a tightly bound ubiquitin adenylate and a covalent Uba1-ubiquitin thiol ester to a conserved active site, Cys632 (28, 29), HsUba1a numbering. Early ATP:PP exchange studies demonstrated that rabbit Uba1 catalyzes an absolutely ordered mechanism in which ATP binding precedes that of ubiquitin prior to the first catalytic step of ubiquitin adenylate formation (29). The activated ubiquitin moiety is subsequently transferred to the thiol ester site comprising Cys632 prior to formation of a second ubiquitin adenylate, to generate the final ternary complex (28, 29). Marked conservation among the activating enzymes for ubiquitin and ubiquitin-like proteins argues that they proceed by similar mechanisms. However, the apparently substoichiometric formation of the predicted Nedd8 adenylate intermediate catalyzed by the reconstituted plant ortholog of AppBp1-Uba3 suggests that the catalytic cycle for Nedd8 activation may exhibit some differ-
ences from that of ubiquitin (30). The latter observation is significant because the presence of enzyme-bound ubiquitin adenylate is required for ubiquitin translocation from the E1 ternary complex to E2 carrier proteins, even though this intermediate is not the immediate donor of activated polyubiquitin (31). To date, the enzymes involved in the activation of ubiquitin-like proteins have not been mechanistically characterized in sufficient detail to resolve these and related questions.

In the present studies, we have found that human erythrocyte protamines represent an excellent source of active AppBp1-Uba3 heterodimer and have utilized covalent affinity purified AppBp1-Uba3 and recombinant human Ubc12 in the first mechanistic studies of Nedd8 activation. The results demonstrate marked conservation between the mechanisms for Nedd8 and ubiquitin activation and identify a critical specificity determinant for polypeptide recognition by their respective activating enzymes.

MATERIALS AND METHODS

Bovine ubiquitin was purchased from Sigma and purified to homogeneity as described previously (32). Homologous wild-type ubiquitin, the recombinant ubiquitin mutant UbR72L (33), and recombinant human Nedd8 were radiolabeled by the chloramine-T method using carrier-free Na14C (34). The [2,8-3H]ATP and Na4[32PP] used in Nedd8/HAdeylate quantitation and ATP-32PP exchange kinetics, respectively, were obtained from PerkinElmer Life Sciences.

Cloning, Expression, and Purification of Human Recombinant Nedd8—Nedd8 was cloned from a HeLa cell cDNA library by PCR amplification using 5′ and 3′ primers flanking the coding sequence that contained Ndel or EcoRI restriction sites, respectively. The PCR product was ligated directly into pGEM-T (Promega) for amplification and purification. The resulting pGEM-Nedd8 construct was digested with NdeI/EcoRI and ligated into similarly restricted pPLhUb to yield a pGEM-Nedd8 construct. The latter polypeptide was expressed in E. coli by induction with 0.1 mm isopropyl-1-thio-β-D-galactoside (IPTG). After 2 h of induction, the cells were collected by centrifugation and lysed by passage through a French press. The lysate was centrifuged at 30,000 × g for 30 min. The resulting pellets were washed with buffer containing 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, and 1 mM DTT, then resuspended to the original lysate volume in the same buffer containing 6 M urea. After being allowed to stand on ice for 30 min, the urea was removed by dialysis against 6 M guanidinium thiocyanate (pH 7.5) (35). Following addition of 50 mM Tris-HCl (pH 7.5) containing 1 mM DTT, insoluble protein was removed by centrifugation prior to applying the dialysate to a 5-ml glutathione-agarose column. Unbound protein was removed by washing the column with 5 bed volumes of 50 mM Tris-HCl (pH 7.5). Bound protein was eluted with 5 bed volumes of 50 mM Tris-HCl (pH 7.5) containing 20 mM glutathione, then concentrated with a Millipore Centricon-100 concentrator and dialyzed against 20 mM Tris-HCl (pH 7.5) containing 1 M urea. The resulting fusion protein was cleaved by digestion with 10 units of thrombin (Amersham Biosciences) using Uba3 for subsequent amplification, purification, and sequencing. Protein concentrations for AppBp1 and Uba3 were estimated densitometrically by comparing Coomassie-stained bands to bovine serum albumin standards.

Cloning and Expression of Human Ubc12—Human Ubc12 was cloned by PCR from a HeLa cell cDNA library using 5′ and 3′ primers immediately flanking the HuUb12 coding sequences that contained Ndel and EcoRI restriction sites, respectively. The PCR product was subcloned into pGEM-T (Promega) for amplification and sequencing. The resulting construct was digested with NdeI and EcoRI, then subcloned into plasmid pGEX-4T-1 to yield pGEX-Ubc12. The AppBp1-Uba3 complex was expressed in E. coli BL21 Lami selecting for ampicillin resistance. Processed AppBp1 or Uba3 were resolved from GST fusion proteins GST-Uba3 and GST-AppBp1 using glutathione affinity chromatography. Bacterial cells containing 50 mM Tris-HCl (pH 7.5) containing 1 mM DTT. Both AppBp1 and Uba3 eluted between 0.31 and 0.35 M NaCl gradient (12.5 mM/min) at 1 ml/min flow rate.

Recombinant AppBp1 and Uba3 proteins were greater than 80% pure as assessed by Coomassie Brilliant Blue staining. Absolute protein concentration was determined spectrophotometrically using the empirical 280/260 nm extinction coefficient of 1.6 (mg/ml)−1 cm−1 (36).

Cloning and Expression of Human AppBp1 and Uba3—The full-length coding sequence of Uba3 was cloned by PCR from human fetal brain expressed sequence tagged I.M.A.G.E. Consortium Clone 45573 (American Type Culture Collection). Flanking primers containing complimentary 5′ and 3′ coding sequences and either Ndel or EcoRI restriction sites, respectively, were used to amplify the cDNA. The resulting PCR product was ligated into pGEM-T (Promega) to yield pGEMT-Uba3 for subsequent amplification, purification, and sequencing. The complete Uba3 coding sequence was then subcloned into pGEX-4T-1 (Amersham Biosciences) using Ndel and EcoRI restriction sites to yield pGEX-Uba3. AppBp1 was cloned by PCR from a HeLa cell cDNA library using flanking primers that contained complimentary 5′ and 3′ coding sequences and either Ndel or EcoRI restriction sites, respectively. The PCR product was ligated directly into pGEM-T. The resulting pGEMT-AppBp1 clone was digested with NdeI and EcoRI, then subcloned into complimentary digested pGEX-4T-1 to yield pGEX-AppBp1. The AppBp1 sequence was verified by sequencing the entire insert.

The glutathione S-transferase fusion proteins GST-Uba3 and GST-AppBp1 were expressed in E. coli BL21 cultures and purified from refolded inclusion bodies by glutathione affinity chromatography. Briefly, bacteria were grown to an A600 of 0.6 at 30 °C and induced by the addition of 0.1 mm isopropyl-1-thio-β-D-galactoside. After 2 h of induction, the cells were collected by centrifugation and lysed by passage through a French press. The lysate was centrifuged at 30,000 × g for 30 min. The resulting pellets were washed with buffer containing 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, and 1 mM DTT, then resuspended to the original lysate volume in the same buffer containing 6 M urea. After being allowed to stand on ice for 30 min, the urea was removed by dialysis against 6 M guanidinium thiocyanate (pH 7.5) to yield 20 mM Tris-HCl (pH 7.5) containing 1 M urea.

Recombinant Uba3 and AppBp1 were estimated densitometrically by comparing Coomassie-stained bands to bovine serum albumin standards.

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The enzyme was expressed in E. coli BL21 cultures and purified from refolded inclusion bodies by glutathione affinity chromatography. Briefly, bacteria were grown to an A600 of 0.6 at 30 °C and induced by the addition of 0.1 mm isopropyl-1-thio-β-D-galactoside. After 2 h of induction, the cells were collected by centrifugation, resuspended in a Millipore Centricon-100 concentrator and dialyzed against 20 mM Tris-HCl (pH 7.5) containing 1 M urea. The resulting fusion protein was cleaved by digestion with 10 units of thrombin (Amersham Biosciences) per milligram of recombinant protein according to the manufacturer’s recommendations. Processed AppBp1 or Uba3 were resolved from GST and thrombin by fast protein liquid chromatography using a Pharmacia Mono Q 5/5 column equilibrated with 50 mM Tris-HCl (pH 7.5) containing 1 mM DTT. Both AppBp1 and Uba3 eluted between 0.31 and 0.35 M NaCl gradient (12.5 mM/min) at 1 ml/min flow rate.

Recombinant AppBp1 and Uba3 proteins were greater than 80% pure as assessed by Coomassie Brilliant Blue staining and resolved by 10% (w/v) SDS-PAGE, and were used without further purification. Protein concentrations for AppBp1 and Uba3 were estimated densitometrically by comparing Coomassie-stained bands to bovine serum albumin standards.

Cloning and Expression of Human Ubc12—Human Ubc12 was cloned by PCR from a HeLa cell cDNA library using 5′ and 3′ primers immediately flanking the HuUb12 coding sequences that contained Ndel and EcoRI restriction sites, respectively. The PCR product was subcloned into pGEM-T (Promega) for amplification and sequencing. The resulting construct was digested with NdeI and EcoRI, then the HuUb12 coding sequence was ligated and ligated into similarly restricted pGEX-4T-1 (Amersham Biosciences) to yield pGEX-HuUb12. The HuUb12 coding sequence was verified by sequencing the entire insert.

The GST-HuUb12 fusion protein was expressed in E. coli BL21 cultures and purified by glutathione affinity chromatography. Bacterial cells transformed with pGEX-HuUb12 were grown at 37 °C to an A600 of 0.6, then protein expression was induced by the addition of isopropyl-1-thio-β-D-galactoside to a final concentration of 1 M. After 2 h of induction, cells were collected by centrifugation, resuspended in buffer containing 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, and 1 mM DTT, then lysed by passage through a French press. The lysate was clarified by centrifugation at 30,000 × g for 30 min and the resulting supernatant was applied to a glutathione-agarose column. Unbound protein was removed by washing the column with 5 bed volumes of 50 mM Tris-HCl (pH 7.5). Bound protein was eluted after dialysis against 50 mM Tris-HCl (pH 7.5) containing 20 mM glutathione, then concentrated with a Millipore Centricon-100 concentrator and dialyzed against 20 mM Tris-HCl (pH 7.5) containing 1 M urea.

The GST-HuUb12 fusion protein was expressed in E. coli BL21 cultures and purified by glutathione affinity chromatography. Bacterial cells transformed with pGEX-HuUb12 were grown at 37 °C to an A600 of 0.6, then protein expression was induced by the addition of isopropyl-1-thio-β-D-galactoside to a final concentration of 1 M. After 2 h of induction, cells were collected by centrifugation, resuspended in buffer containing 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, and 1 mM DTT, then lysed by passage through a French press. The lysate was clarified by centrifugation at 30,000 × g for 30 min and the resulting supernatant was applied to a glutathione-agarose column. Unbound protein was removed by washing the column with 5 bed volumes of 50 mM Tris-HCI

2 The identical aromatic amino acid content for ubiquitin and human Nedd8 allowed us to use the empirical extinction coefficient of the former polypeptide in this spectrophotometric assay.
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(pH 7.5), 2 mM EDTA, and 1 mM DTT. Bound protein was eluted with 50 mM Tris-HCl (pH 7.5) containing 20 mM glutathione, then concentrated using a Millipore Ultrafree BioMax-5K centrifugal filter. Following processing of the fusion protein with thrombin (Amersham Biosciences) at 10 units per mg of fusion protein, free HsUbc12 was further purified by fast protein liquid chromatography using a Pharmacia Mono Q 5/5 column equilibrated with 50 mM Tris-HCl (pH 7.5) containing 1 mM DTT. Elution of purified recombinant protein was eluted with a 0.5 M NaCl gradient (12.5 mM/min) at 1 mL/min. The protein was greater than 95% pure as assessed by Coomassie Blue Brilliant staining. The concentration of active protein was determined by an end point thiol ester assay using [125I]-Nedd8 (about 10,000 cpm/pmol) and affinity purified AppBp1-Uba3 heterodimer (37). Purified protein was flash frozen and stored at −80 °C for several months without loss of activity.

-**Initial Studies with Recombinant AppBp1 and Uba3—**Recombinant AppBp1 and Uba3 were expressed separately and purified as described under “Materials and Methods.” The purified proteins were of the predicted molecular weights as assessed by SDS-PAGE and Coomassie staining (data not shown). Neither of the two purified proteins alone at 20 nM demonstrated a detectable level of [125I]-Nedd8 thiol ester formation by non-reducing SDS-PAGE nor was such a thiol ester detected when 20 nM of each of the two proteins were mixed (not shown). Similarly, there was no detectable Nedd8 [3H]Adenylation formed by either of the recombinant proteins individually at 20 nM or in equimolar combination. However, equimolar mixtures of AppBp1 and Uba3 (20 nM) catalyzed a low rate of Nedd8-dependent ATP:32PPi exchange that was not observed with either subunit alone (not shown). Because ATP:[32PPi exchange must proceed through a Nedd8 adenylation intermediate (29, 39), a low level of active heterodimer must be formed on mixing the recombinant subunits which was below the limit of detection by the stoichiometric Nedd8 [3H]Adenylation assay yet detectable by the much more sensitive isotope exchange rate assay. The reconstituted AppBp1-Uba3 heterodimer must also form a Uba3-[125I]-Nedd8 thiol ester because a low but measurable rate of [125I]-Nedd8 transthiolation to Ubc12 was also found at 20 nM recombinant AppBp1 and Uba3 (not shown).

In refolding experiments, equimolar amounts of the recombinant proteins were combined and urea was added to a final concentration of 6 M. The urea was then removed by dialysis to allow refolding of the proteins. Refolding the combined proteins did not enhance the activity of [125I]-Nedd8 transthiolation to Ubc12, as a measure of functional heterodimer formation, above that found by combining the separately refolded subunits. However, a time-dependent, 10-fold increase in the rate of [125I]-Nedd8 transthiolation to HsUbc12 was noted when equimolar native AppBp1 and Uba3 were combined and then preincubated at 37 °C prior to initiating the assay. The time-dependent increase in HsUbc12 transthiolation activity followed first-order kinetics with a t1/2 of 9.8 min. Together, these observations suggest that the in vitro formation of an active AppBp1-Uba3 heterodimer is relatively slow.

Affinity Purification of the AppBp1-Uba3 Heterodimer from Human Erythrocytes—Because human erythrocytes are a rich source of the ubiquitin-activating enzyme, we tested human erythrocyte Fraction II for Nedd8 activating activity in an effort to obtain a more practical source of functional AppBp1-Uba3 heterodimer. Fraction II from human erythrocytes shows a pronounced band of [125I]-Nedd8 thiol ester when incubated with the radiolabeled polypeptide in the presence (lane 2) but not the absence (lane 1) of ATP (Fig. 1A). The mobility of the [125I]-Nedd8 thiol ester band on SDS-PAGE gave an apparent...
When the human erythrocyte Fraction II was passed through an Affi-Gel 10 Nedd8 affinity column, the activity forming Uba3 thiol ester was depleted from the unadsorbed Fraction II (Fig. 1A, lane 3). Approximately 28% of the initial activity forming Uba3 thiol ester activity was recovered when the column was eluted with 2 mM AMP and PP′ (Fig. 1A, lane 4) and 58% of the initial activity forming Uba3 thiol ester activity was recovered in the pH 9.0–10 mM DTT eluate (Fig. 1A, lane 5). Resolution of the AMP-PP′ and pH 9-DTT eluted samples by reducing SDS-PAGE followed by silver staining revealed two bands of 62 and 51 kDa that were in good agreement with the expected molecular weights of 63,000 and 49,000 for human AppBp1 and Uba3, respectively (Fig. 1B). Interestingly, at higher sample loads we noted that the AppBp1 band showed a markedly lower color yield following Coomassie staining than did the Uba3 band, leading to the erroneous impression that the subunits are present at a non-stoichiometric ratio (not shown). However, subsequent silver staining of the gel showed approximately identical intensities for the two subunits (Fig. 1B), consistent with a 1:1 ratio for active heterodimer. This conclusion was confirmed by a similar difference in color yield on Coomassie staining of normalized thrombin-processed recombinant GST-AppBp1 and GST-Uba3 (not shown). In addition, a 1:1 stoichiometry for AppBp1-Uba3 is consistent with the crystal structure of the heterodimer (27).

The AppBp1-Uba3 Heterodimer Forms a Stoichiometric Nedd8 Ternary Complex—Ubiquitin-activating enzyme forms a ternary complex during the activation of ubiquitin that is composed of 1 eq each of tightly bound ubiquitin adenylate and covalent ubiquitin thiol ester (28, 29). Human AppBp1-Uba3 heterodimer catalyzes an analogous ATP:32PP′ exchange reaction that is absolutely dependent on the presence of Nedd8 (not shown). At 1 μM Nedd8 and 1 mM 32PP′, the concentration dependence of ATP on the initial rate for human erythrocyte AppBp1-Uba3 heterodimer-catalyzed ATP:32PP′ exchange has been used to demonstrate that ubiquitin-activating enzyme proceeds through an ordered addition mechanism for which ATP is the leading and ubiquitin the trailing substrate (28, 29). To determine whether human erythrocyte AppBp1-Uba3 heterodimer forms a similar Nedd8 ternary complex, the stoichiometry of Nedd8 [3H]adenylate and Uba3-[125I]-Nedd8 thiol ester was determined in parallel with a quantity of human erythrocyte Uba3 ubiquitin-activating enzyme that produced a silver-stained band following SDS-PAGE resolution of the same intensity as affinity purified human erythrocyte Uba3. Table I demonstrates that both human Uba1 and AppBp1-Uba3 heterodimer form amounts of ubiquitin [3H]adenylate and Nedd8 [3H]adenylate, respectively, that are stoichiometric with their corresponding levels of radioiodinated thiol ester intermediate. The ~33% difference in absolute quantities of the intermediates formed for equivalent amounts of HsUba1 and HsUba3 likely reflect differences in the relative amounts of active protein. The data of Table I represent the first demonstration that AppBp1-Uba3 catalyzes formation of a stable, stoichiometric enzyme-bound Nedd8 adenylate intermediate. Therefore, the stoichiometry for ternary complex formation by human AppBp1-Uba3 heterodimer is conserved with respect to that for HsUba1.

| AppBp1-Uba3 Heterodimer Catalyzes a Random Addition Mechanism for Nedd8 Activation | Previously, ATP:32PP′ exchange kinetics have been used to demonstrate that ubiquitin-activating enzyme proceeds through an ordered addition mechanism for which ATP is the leading and ubiquitin the trailing substrate (29). Human AppBp1-Uba3 heterodimer catalyzes an analogous ATP:32PP′ exchange reaction that is absolutely dependent on the presence of Nedd8 (not shown). At 1 μM Nedd8 and 1 mM 32PP′, the concentration dependence of ATP on the initial rate for human erythrocyte AppBp1-Uba3 heterodimer-
catalyzed ATP-32PPi exchange shows normal hyperbolic kinetics (not shown). However, at 1 mM ATP and PPi, the concentration dependence with respect to Nedd8 exhibits substrate inhibition at concentrations above about 2 μM (Fig. 2). Ubiquitin-activating enzyme exhibits an analogous substrate inhibition at a similar range of ubiquitin concentrations that reflects ordered substrate binding (29). At Nedd8 concentrations below 1 μM, the dependence of the initial rate for isotope exchange on [Nedd8], is hyperbolic and yields a linear double reciprocal plot (not shown). Non-linear hyperbolic fitting of initial rate data within the linear region of the reciprocal plot yielded values for Vmax and K1/2 of 54 ± 1.4 pmol/min and 0.83 ± 0.05 μM, respectively. Because Vmax = kcat [AppBp1-Uba3], the corresponding value for kcat of 1.6 ± 0.1 s−1 was calculated from the stoichiometric formation of the AppBp1-Uba3 ternary complex as a functional activity assay for the heterodimer. At concentrations above 100 μM, the ATP-32PPi exchange rates tend to a limiting value of 2.2 pmol/min, representing 4% of the extrapolated Vmax of 54 ± 1.4 pmol/min. That the initial isotope exchange rate tends to a limiting value rather than zero at infinite Nedd8 concentration indicates that the mechanism for the AppBp1-Uba3 heterodimer follows a formally random addition mechanism, although there is a preferential order of ATP binding preceding that of Nedd8 based on their relative affinities (41).

**Transthiolation Kinetics for AppBp1-Uba3 Heterodimer**—Work from our laboratory has recently shown that initial rates for the transfer of 125I-ubiquitin thiol ester from the E2 ternary complex to various E2 isozymes can be used as a facile kinetic assay for determining the intrinsic Kd of substrate binding (38). Fig. 3 shows an analogous double reciprocal plot for the dependence of 125I-Nedd8 concentration on the initial rate of HsUbc12 transthiolation catalyzed by the human AppBp1-Uba3 heterodimer. Linearity of the plot demonstrates that the heterodimer conforms to simple hyperbolic kinetics with respect to radiolabeled Nedd8. In addition, observation of strict hyperbolic kinetics over a Nedd8 concentration range for which substrate inhibition is observed for ATP-32PPi exchange (Fig. 2) confirms that the latter behavior is a consequence of pseudo-ordered substrate addition rather than formation of a nonproductive dead end complex. When fitted by non-linear hyperbolic regression analysis, the data of Fig. 3 yields a Kd value of 0.95 ± 0.18 μM for human AppBp1-Uba3 ortholog by direct transthiolation kinetics (38). Similar affinities for Nedd8 binding to human AppBp1-Uba3 and ubiquitin binding to HsUbc12 transthiolation catalyzed by the human AppBp1-Uba3 heterodimer.

The dependence of the initial rate for 125I-Nedd8 trans-thiolation with respect to changes in ATP and HsUbc12 concentrations exhibited similar hyperbolic kinetics based on the linearity of their respective double reciprocal plots (not shown). Values of Kd and Vmax, the latter yielding corresponding estimates for kcat, were calculated from non-linear hyperbolic fitting of the data and are summarized in Table II. The Kd of 103 ± 12 μM for ATP binding to AppBp1-Uba3 (Table II) was considerably higher than the Kd of 7.0 ± 1.1 μM recently reported for ATP binding to rabbit Uba1 ortholog by direct transthiolation kinetics (38). The latter correspondences in affinities for Nedd8 binding to human AppBp1-Uba3 and ubiquitin binding to HsUbc12 transthiolation catalyzed by the human AppBp1-Uba3 heterodimer are remarkably close to the values of 4.5 ± 0.3 s−1 and 4.8 ± 0.2 s−1 recently reported for HsUbc2b transthiolation catalyzed by human and rabbit Uba1 orthologs, respectively (38). Concordance in the kcat values for the ubiquitin- and Nedd8-specific enzyme para-
logs presumably reflects similar geometries for the transition states of the respective transthiolelation reactions.

Ala<sup>72</sup> Is an Important Specificity Determinant for Nedd8 Recognition by AppBp1-Uba3 Heterodimer—Of the known ubiquitin-like proteins, Nedd8 is the most similar (58% identity) in sequence to ubiquitin (10); therefore, we were interested in whether ubiquitin could substitute for Nedd8 in the catalytic cycle of the AppBp1-Uba3 heterodimer. In stoichiometry studies similar to those of Table I, we were unable to detect formation of either heterodimer-bound ubiquitin [<sup>3</sup>H]adenylate or covalent Uba3-<sup>125</sup>I-ubiquitin thiol ester (not shown). In addition, the more sensitive turnover assay involving heterodimer-catalyzed HsUbc12 transthiolelation described under “Materials and Methods” failed to detect any HsUbc12-<sup>125</sup>I-ubiquitin thiol ester formation after 3 min incubation in the presence of 66 μM <sup>125</sup>I-ubiquitin (5000 cpm/μmol) and 20 nM heterodimer. Therefore, AppBp1-Uba3 heterodimer appears to exhibit marked discrimination against ubiquitin as an alternative substrate.

Because wild type ubiquitin is not activated by human AppBp1-Uba3 heterodimer, we tested ubiquitin as a competitive inhibitor of <sup>125</sup>I-Nedd8 activation in a coupled HsUbc12 transthiolelation assay under AppBp1-Uba3 limiting conditions. In assays conducted as described under “Materials and Methods” in the presence of 0.2 mM human AppBp1-Uba3 heterodimer, 1 μM HsUbc12, and 1 μM <sup>125</sup>I-Nedd8, we observed 14% inhibition in the initial rate of HsUbc12 transthiolelation in the presence of 100 μM wild type ubiquitin. Reasonably assuming competitive inhibition, the observed 14% inhibition corresponds to a <i>K<sub>i</sub></i> (measured as <i>K<sub>i</sub></i>) for wild type ubiquitin of about 300 μM representing a ΔΔG° of about 3.4 kcal/mol.

Docking studies of Nedd8 with human AppBp1-Uba3, modeled after the analogous interaction of MoaD with MoeB (21), has prompted Walden et al. (27) recently to suggest that Ala<sup>72</sup> of Nedd8 is a critical specificity determinant allowing the heterodimer to distinguish its cognate polypeptide substrate from other ubiquitin-like paralogs. The ability of wild type Nedd8 to support AppBp1-Uba3 catalyzed activation and HsUbc12 transthiolelation is significantly ablated by mutating the Uba3 residues Leu<sup>206</sup> and Tyr<sup>207</sup> predicted to interact with Ala<sup>72</sup> of Nedd8 (27). We have previously used the UbR72L point mutant to show that Arg<sup>72</sup> is an important binding determinant for ubiquitin recognition by rabbit reticulocyte Uba1 (33). Although wild type <sup>125</sup>I-ubiquitin is unable to support measurable AppBp1-Uba3 activation or HsUbc12 transthiolelation, the data of Fig. 4 demonstrate that the <sup>125</sup>I-ubiquitin thiol ester formation with respect to <sup>125</sup>I-UbR72L, is hyperbolic, demonstrated by the linearity of the reciprocal plot in Fig. 4, and yields a <i>K<sub>m</sub></i> of 20 ± 9 μM by nonlinear regression analysis. In addition, there is remarkable concordance between the <i>k<sub>cat</sub></i> for <sup>125</sup>I-UbR72L of 0.9 ± 0.3 s<sup>-1</sup> calculated from the <i>V<sub>max</sub></i> (Fig. 4) and the <i>k<sub>cat</sub></i> of 3.5 ± 0.2 s<sup>-1</sup> for <sup>125</sup>I-Nedd8 (Table II). The good agreement suggests that specificity is principally an affinity effect and that the transthiolelation step from the AppBp1-Uba3 ternary complex to HsUbc12 otherwise exhibits little discrimination between the two orthologs compared with the initial step of polypeptide binding.

### DISCUSSION

The conjugation of ubiquitin and related ubiquitin-like polypeptides to specific protein targets represents a fundamental and highly conserved strategy of eukaryotic cell regulation (43–45). These post-translational modifications require distinct yet evolutionarily related enzyme pathways that share a common mechanism in which the half-reactions of activation and ligation are catalyzed by separate enzymes (42, 43). The Uba1 ubiquitin-activating enzyme catalyzes the first step in the conjugation of ubiquitin to protein targets and serves as the archetype for similar steps in the activation of other ubiquitin paralogs that now include Sumo, Nedd8, Huh1, ISG15, FAT10, and Apg12 (10). The marked sequence homology between Uba1 and the AppBp1-Uba3 heterodimer required for Nedd8 activation reveals a divergent evolutionary relationship; however, because no activation step for a ubiquitin-like protein has been examined in detail, it has been uncertain whether the similarity in sequences is mirrored by a shared catalytic mechanism.

The present studies demonstrate that the marked sequence homology between Uba1 and AppBp1-Uba3 reflects an overall conservation in mechanism. Quantitative stoichiometry studies show for the first time that human AppBp1-Uba3 heterodimer forms a stable ternary complex comprised of equivalent amounts of Nedd8 adenylate and Uba3-Nedd8 thiol ester (Table I). This complex is analogous to the ternary complex originally observed for Uba1 (28). Earlier detection of only trace Rub1 [<sup>32</sup>P]adenylate formation by the plant heterodimer ortholog Axr1-Ecr1 (30) presumably reflects the exceedingly low yield of active heterodimer formed when reconstituted from individual recombinant subunits (this study). The latter conclusion is supported by the extensive hydrophobic interface between AppBp1 and Uba3 revealed in the recent crystal structure of the human heterodimer (27). Time-dependent reconstitution of Nedd8 activating activity, when monitored by the initial rate of HsUbc12 transthiolelation, upon mixing of the separate subunits (this study) most likely reflects a rapid initial subunit association followed by a slower reorganization to yield the native heterodimer.
In addition to the conservation in formation of a stable ternary complex, human AppBp1-Uba3 heterodimer is characterized by a pseudo ordered mechanism of substrate binding with ATP serving as the obligatory leading and ubiquitin the obligatory trailing substrate, based on early kinetic isotope exchange studies (29). In such studies, ordered addition is characterized by substrate inhibition at high concentrations of the trailing ligand and a limiting rate tending to zero velocity at infinite concentration, discussed in Ref. 29. In the present study, the dependence of initial ATP-\textsuperscript{32}PP exchange rate on [ATP], yields a hyperbolic dependence (not shown) while that for [Nedd8], exhibits substrate inhibition (Fig. 2). Therefore, human AppBp1-Uba3 heterodimer proceeds through a preferentially ordered binding of ATP followed by Nedd8 that resembles that of Uba1. However, because the limiting initial rate at high Nedd8 concentrations tends to a value of 2.2 pmol/min, representing about 4% of the extrapolated \( V_{\text{max}} \) of 54 ± 1.4 pmol/min, the mechanism of human AppBp1-Uba3 is formally random. Burch and Haas (33) have previously shown that Uba1-dependent activation of a UbR72L point mutant occurs through a random substrate addition mechanism. More recent alanine scanning mutagenesis of ubiquitin identified several additional surface residues that result in purely random addition or pseudo ordered addition, as shown here for the AppBp1-Uba3 catalyzed activation of Nedd8.3 These observations indicate that ordered addition is not a structural requisite for the catalytic competence of ubiquitin-activating enzyme but reflects differential binding affinity for ATP and ubiquitin as leading versus trailing substrates. The pseudo ordered mechanism of human AppBp1-Uba3 suggests the relative affinities for ATP versus Nedd8 as leading versus trailing substrate are less constrained than for Uba1 and wild type ubiquitin.

Because formation of the AppBp1-Uba3 ternary complex is rapid, direct kinetic studies of substrate binding is technically challenging. However, by exploiting the HsUbc12 transthiolation reaction as a coupled reporter assay, we have been able for the first time to quantitate the affinity of substrate binding to human AppBp1-Uba3 heterodimer. As noted earlier, the affinity of Nedd8 for human AppBp1-Uba3 heterodimer \( (K_m = 0.95 \pm 0.18 \mu M) \) is remarkably similar to the \( K_m \) of 0.58 \( \mu M \) found for ubiquitin binding to rabbit Uba1 in equilibrium studies (29) and the \( K_m \) of 0.8 ± 0.2 \( \mu M \) recently determined from analogous HsUba1-catalyzed HsUbc2b transthiolation kinetics (38). Likewise, the \( K_m \) of 43 ± 13 \( \mu M \) for HsUbc12 binding to AppBp1-Uba3 heterodimer is in the range of the \( K_m \) of 123 ± 19 \( \mu M \) for HsUbc2b binding to human ubiquitin-activating enzyme (38). The marked concordance in these substrate affinities probably reflects selective constraints imposed by the steady state concentrations of these ligands within the cell to prevent ubiquitin or Nedd8 activation from becoming rate-limiting in their respective ligation pathways, discussed in Ref. 42.

In contrast, we consistently found that the \( K_m \) for ATP binding to human AppBp1-Uba3 heterodimer (103 ± 12 \( \mu M \), Table II) was considerably larger than the \( K_m \) of 7.0 ± 1.1 \( \mu M \) for binding of the nucleotide to HsUba1 (38). The potential functional consequence of this difference is obscure because cellular ATP concentrations generally range near 5 \( \mu M \). The crystal structure for the \textit{E. coli} MoeB-ATP-MoaD ternary complex (21) and ATP docking studies to human AppBp1-Uba3 that was based on the MoeB-ATP-MoaD coordinates (27) identify 12 residues within the conserved nucleotide binding pocket that potentially interact with ATP. In a closer examination of the MoeB-ATP-MoaD structure we find an additional residue, Asp\textsuperscript{506}, that is well positioned to hydrogen bond to the ribose ring of ATP or engage in a charge interaction with the ATP-chelated Mg\textsuperscript{2+}. Of these 13 residues, 10 are absolutely conserved among MoeB, human Uba3, the corresponding human Sumo-activating enzyme subunit Uba2, and the human ubiquitin-activating Uba1 (27). Among the remaining three variant positions, all of which interact with the adenine ring (21, 27), Uba3 contains Ile\textsuperscript{54} in place of the invariant valine present within the other three activating enzyme paralogs; the Ile\textsuperscript{127} that is conserved among Uba3, Uba2, and MoeB is replaced by Val\textsuperscript{552} in Uba1; and Ser\textsuperscript{147} of Uba3 replaces a conserved asparagine in the other three enzymes. Presumably one or more of these substitutions account in part for the observed difference in ATP binding affinity (\( \Delta G^o = 0.7 \) kcal/mol) between AppBp1-Uba3 and Uba1.

The fidelity of signaling by conjugation of Class I ubiquitin-like proteins requires absolute specificity with respect to the polypeptide, as initially suggested for ISG15 (46). Discrimination among the ubiquitin-like proteins must occur at their respective activation steps because the polypeptides are committed to a specific ligation pathway once charge onto the cognate Ubc carrier protein. The present data demonstrates quantitatively that AppBp1-Uba3 exhibits absolute specificity for Nedd8 over ubiquitin. Catalytic specificity is expressed as \( k_{\text{cat}}/K_m \), the effective second order rate constant. The kinetics for AppBp1-Uba3-catalyzed Nedd8 transthiolation of HsUbc12 yields a \( k_{\text{cat}}/K_m \) of \( 3.5 \times 10^5 \) M\textsuperscript{-1}s\textsuperscript{-1} (Table II). Although 125I-ubiquitin fails to support HsUbc12 thiol ester formation catalyzed by AppBp1-Uba3 heterodimer, the lower limit of detection from the kinetic study (about 10 cpm) allows us to estimate a \( k_{\text{second order}} \leq 700 \) M\textsuperscript{-1}s\textsuperscript{-1} for wild type ubiquitin. Therefore, AppBp1-Uba3 heterodimer exhibits a catalytic specificity \( \geq 500 \)-fold for Nedd8 versus wild type ubiquitin. The data of Fig. 4 requires a \( k_{\text{cat}}/K_m \) of \( 4.2 \times 10^3 \) M\textsuperscript{-1}s\textsuperscript{-1} for 125I-UbR72L, reducing the difference in specificity to 83-fold through the contribution at residue 72. Whitby et al. (47) has shown that Arg\textsuperscript{72} is also critical in allowing the ubiquitin-activating enzyme to discriminate between ubiquitin and Nedd8. Wild type Nedd8 exhibits low affinity binding to rabbit reticulocyte Uba1 (apparent \( K_m = 182 \pm 47 \mu M \); however, a Nedd8A72R point mutant binds the activating enzyme in competitive Uba1-125I-ubiquitin thiol ester assays with an apparent \( K_m \) of 2.8 ± 0.2 \( \mu M \) that is nearly identical to the apparent \( K_m \) of 2.0 ± 0.2 \( \mu M \) for unlabeled ubiquitin (47).

The present studies are the first comprehensive examination of the enzymology for Nedd8 activation catalyzed by human AppBp1-Uba3. The data demonstrate quantitatively that the marked sequence conservation between the Nedd8-specific heterodimer and the Uba1 ubiquitin-activating enzyme is mirrored by a conservation in mechanism that includes ternary complex stoichiometry and substrate affinities. In addition, the studies show that human erythrocytes represent a practical source for the facile isolation of this important enzyme reagent.

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