Mechanistic Studies on Activation of Ubiquitin and Di-ubiquitin-like Protein, FAT10, by Ubiquitin-like Modifier Activating Enzyme 6, Uba6*§

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Background: The Uba6 pathway and its components play an important role in a variety of biological processes.

Results: The mechanism of how Uba6 activates two distinct substrates, ubiquitin and FAT10, was characterized.

Conclusion: Uba6 was shown to use a similar mechanism for activating both substrates with a greater affinity for FAT10.

Significance: Relative levels of ubiquitin and FAT10 could regulate the Uba6 pathway in cells.

Uba6 is a homolog of the ubiquitin-activating enzyme, Uba1, and activates two ubiquitin-like proteins (UBLs), ubiquitin and FAT10. In this study, biochemical and biophysical experiments were performed to understand the mechanisms of how Uba6 recognizes two distinct UBLs and catalyzes their activation and transfer. Uba6 is shown to undergo a three-step activation process and form a ternary complex with both UBLs, similar to what has been observed for Uba1. The catalytic mechanism of Uba6 is further supported by inhibition studies using a mechanism-based E1 inhibitor, Compound 1, which forms covalent adducts with both ubiquitin and FAT10. In addition, pre-steady state kinetic analysis revealed that the rates of UBL-adenylate (step 1) and thioester (step 2) formation are similar between ubiquitin and FAT10. However, distinct kinetic behaviors were also observed for ubiquitin and FAT10. FAT10 binds Uba6 with much higher affinity than ubiquitin while demonstrating lower catalytic activity in both ATP-PP i exchange and E1-E2 thioleolation assays. Also, Compound 1 is less potent with FAT10 as the UBL compared with ubiquitin in ATP-PP i exchange assays, and both a slow rate of covalent adduct formation and weak adduct binding to Uba6 contribute to the diminished potency observed for FAT10. Together with expression level analysis in IM-9 cells, this study sheds light on the potential role of cytokine-induced FAT10 expression in regulating Uba6 pathways.

In eukaryotic cells, post-translational modification of proteins and lipids by ubiquitin and ubiquitin-like proteins (UBLs) is involved in a wide range of biological processes, including protein turnover via the proteasome or autophagy, protein localization, antimicrobial functions, and tRNA thiolation among others (1, 2). Covalent attachment of UBLs to their substrates is catalyzed by an enzymatic cascade consisting of an E1 (activating enzyme), an E2 (conjugating enzyme), and sometimes an E3 (ligase). In humans, eight E1s have been identified that can be grouped into two classes based on sequence homology and domain organization (1, 3). The canonical E1s exist either as monomers or heterodimers and include ubiquitin-activating enzyme (UAE, also known as Ube1), SUMO-activating enzyme, NAE, Uba6, and Uba7. Canonical E1s harbor a single functional adenylation domain, a large catalytic cysteine domain, and a ubiquitin-fold domain (UFD) (1). Uba4, Uba5, and ATG7 are considered noncanonical E1s in that they form homodimers, possess smaller catalytic Cys domains, and are involved in divergent domain structures (1). So far, 17 UBLs belonging to nine distinct classes have been identified in humans (4).

Despite their structural and functional diversity, E1s are proposed to use a conserved, multistep process for UBL activation and transfer to an E2 (1, 5). The mechanism of ubiquitin activation by UAE serves as a model for understanding other E1 enzymes (6, 7). Haas et al. (6, 7) demonstrated that activation of ubiquitin by UAE involves three steps (Fig. 1A). In step 1, UAE binds ATP and ubiquitin and catalyzes ubiquitin adenylation formation with release of pyrophosphate. In step 2, nucleophilic attack of the adenylate intermediate by the conserved cysteine in the catalytic cysteine domain leads to a UAE-S~ubiquitin thioester (where ~ represents the thioester bond between the catalytic cysteine of UAE and the C-terminal glycine of ubiquitin) with AMP as the by-product. In step 3, UAE binds a second ATP and ubiquitin to form ubiquitin adenylate, similar to step 1. The resulting UAE-ubiquitin ternary complex contains two ubiquitin molecules, one as the adenylate and the other covalently attached to the catalytic cysteine, and is competent for transferring ubiquitin to a cysteine residue on an E2 via a transthiolation reaction (6–9). Binding of ATP in the E1 adenyltransferase site is sufficient for E1-E2 transthiolation (8). Interestingly, in experiments in which UAE was charged with NEDD8, a UBL that shares ~60% sequence

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§ This article contains two Figs. S1–S5 and Table S1.

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2 The abbreviations used are: UBL, ubiquitin-like protein; NAE, NEDD8-activating enzyme; UFD, ubiquitin-fold domain; SPR, surface plasmon resonance; HTRF, homogeneous time-resolved fluorescence; NEM, N-ethylmaleimide; BisTris, 2-[bis(2-hydroxyethyl)amino]2-(hydroxymethyl)propane-1,3-diol.
homology with ubiquitin, it was shown that NEDD8 was efficiently transferred to UAE-ubiquitin pathway specific E2s (10, 11). Furthermore, it was also found that E1 enzymes specifically bind their cognate E2 through specific interactions in their β-grasp domains irrespective of the UBL (12). This suggested that the UBL pathway specificity is determined by cognate recognition of UBL and E2 by the E1 and that there is no intrinsic discrimination against noncognate UBLs in E1-E2 transfer or subsequent steps (11).

Recently, Uba6 was identified as a second E1 in mammals that can efficiently activate ubiquitin (13–15). Interestingly, Uba6 was also found to be uniquely capable of activating a second UBL called FAT10 (13). FAT10, human leukocyte antigen F-associated transcript 10, is an 18-kDa protein that contains two ubiquitin-like domains that share 29 and 36% sequence identity with ubiquitin, respectively (16). The expression of FAT10 is induced by tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) (17). Furthermore, USE1, an E2 that interacts exclusively with Uba6 (14), has also been shown to accept both ubiquitin and FAT10 from Uba6 (18). In addition, in vitro and in vivo studies have revealed that the Uba6 pathway and its components play an important role in a variety of biological processes, including ubiquitination via the N-end rule pathway, proteasomal degradation, NF-κB activation, p53 transcriptional activation, and solubilizing polyglutamine proteins (19–23).

Here, we sought to understand the mechanistic basis by which Uba6 recognizes and activates ubiquitin and FAT10, two structurally diverse UBLs. In this study, we demonstrate that Uba6 undergoes a three-step activation process and forms a ternary complex with both FAT10 and ubiquitin, similar to what has been observed for UAE and ubiquitin. The catalytic mechanism of Uba6 is further supported by inhibition studies using a mechanism-based E1 inhibitor, Compound 1, that forms covalent adducts with both ubiquitin and FAT10 (24, 25). Detailed binding, steady state, and pre-steady state kinetic analysis revealed similarities and differences between ubiquitin and FAT10 in Uba6-catalyzed transformations. Together with UBL expression analysis in IM-9 cells, this study provides a biochemical framework for understanding how UBL selectivity and the relative abundance of ubiquitin and FAT10 in cells may affect Uba6 pathway function.

**EXPERIMENTAL PROCEDURES**

**Materials**—[32P]PPi (catalog no. NEX019) and [α-32P]ATP (catalog no. BLU003H250UC) were obtained from Perkin-Elmer Life Sciences. Bovine untagged ubiquitin (catalog no. U6253) was purchased from Sigma. Other chemicals were purchased from Sigma. N-terminal FLAG-tagged ubiquitin, FAT10, and other UBL proteins were generated by gene synthesis and subcloning in a pDEST14 vector and were expressed in *Escherichia coli*. Mouse monoclonal anti-FLAG M2 antibody was purchased from Sigma (catalog no. F1804). Rabbit monoclonal anti-Compound 1 antibody was generated as described previously (24, 26). Rabbit monoclonal anti-USE1 and anti-Uba6 antibodies were generated by Millenium. N-terminally His6-tagged human Uba6 (and other E1s) and N-terminally glutathione S-transferase (GST)-tagged Uba6 fusion protein were expressed in S9 insect cells and purified as described (26). USE1 was expressed and purified as an N-terminally GST-tagged fusion protein in *E. coli*, followed by treatment of AcTEV™ protease (Invitrogen) to remove the GST tag. Expressed proteins were purified by affinity or conventional chromatography using standard buffers. Stock concentrations of E1 and E2 proteins were determined spectrophotometrically as described (27). Purified ubiquitin-Compound 1 adduct was generated as described by Chen et al. (25).

**ATP-PPi Exchange Assay**—The ATP-PPi exchange assay was performed using a modified protocol developed by Bruzzone et al. (27). For titrations and *K*₅₀ determination, substrates were serially diluted in 1:2 or 1:3 across a 96-well plate. All reaction mixtures contained 5 nM Uba6 and were incubated at 30 °C in 1× E1 buffer (50 mM HEPES, pH 7.5, 25 mM NaCl, 10 mM MgCl₂, 0.05% BSA, 0.01% Tween 20, and 1 mM DTT) for 30 min. For the UBL titrations, 1 mM ATP and 0.2 mM PPi (50 cpm/pmol [32P]PPi) were used. For ATP and PPi, titrations, the UBL concentration was fixed at 4 μM. In exchange kinetics, the concentration of substrate yielding the half-maximal rate is not equivalent to *K*₅₀; therefore *K*₅₀ was used instead (7). *K*₅₀ values were estimated by fitting the data using the Michaelis-Menten equation.

For *K*₅₀ determinations, 1.25, 2.5, or 5 nM Uba6 was incubated at 30 °C for various time points in 1× E1 buffer with 4 μM UBL, 1 mM ATP, and 0.5 mM PPi (50 cpm/pmol [32P]PPi). *K*₅₀ values were calculated using rates of the reactions and an [α-32P]ATP standard curve.

For potency measurement, Compound 1 or ubiquitin-Compound 1 was serially diluted into a 96-well assay plate and a mixture containing 5 nM Uba6, 20 μM ATP, 200 μM PPi (50 cpm/pmol [32P]PPi), and 1 μM UBL. Reactions were run for 60 min at 30 °C.

For reversibility studies, UBL-Compound 1 adduct was made on Uba6 by incubating 500 nM Uba6 with 2 μM UBL, 10 μM ATP, 200 μM Compound 1 in 10 mM MgCl₂, 2 mM DTT, 0.05% BSA, 50 mM HEPES, pH 7.5, for 30 min at room temperature. Excess Compound 1 was removed by spinning samples over a pre-equilibrated ZEBA desalting column (Thermo Scientific, catalog no. 89883) at 2000 relative centrifugal force for 2 min. Spun samples were diluted in 1 in 100 into a reaction mixture to initiate the ATP-PPi exchange reaction. The final reaction mixture contained 5 nM Uba6, 2 μM ubiquitin, 1 mM ATP, 200 μM PPi (50 cpm/pmol [32P]PPi) in 1× E1 buffer. Reactions were incubated at 30 °C and stopped at several time points between 0 and 240 min.

**Thin Layer Chromatography (TLC) AMP Assay**—For AMP turnover analysis, reaction mixtures contained 1 μM wild-type or C625A Uba6, 10 μM UBL, 25 μM ATP ([α-32P]ATP (1 μCi/nmol)), 100 μM Compound 1 (where indicated), 5 mM MgCl₂ in 1× E1 buffer. Reactions, in duplicate, were incubated at 30 °C for 5 min before being stopped with 25 mM EDTA. The reaction mixture (1 μl) was then spotted onto a PEI-TLC plate (J. T. Baker, Inc., catalog no. 4474-04) that had been pretreated with water followed by 1 M LiCl. The [32P]-labeled AMP and
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ATP was quantified radiometrically using a phosphorimager as described previously (25).

Binding Affinity Measurement between Uba6 and Ubiquitin or FAT10 Using Surface Plasmon Resonance (SPR)—SPR experiments were performed on a Biacore S11 instrument (GE Healthcare) using a similar procedure as described before (25). N-terminal GST-tagged Uba6 and GST protein were immobilized on a sensor chip surface using the anti-GST antibody capturing method described previously (25). The SPR data were collected at 25 °C with a flow rate of 90 μl/min in a sample running buffer containing 10 mM HEPES, 150 mM NaCl, 0.005% P-20 (as surfactant), 0.1 mg/ml BSA, pH 7.5. All data acquisition (in duplicate) and subsequent analysis were performed with recombiant GST as the control. The kinetics of association and dissociation data were fit with a single exponential rise or decay equation. The equilibrium affinity binding data were fit using a one-site binding model.

Competition of UBL-Compound 1 Adduct Formation by Western Blot Assay—Fixed concentrations of untagged UBL (0.5 μM final) were added to tubes containing 20 μM Compound 1. Next, the second UBL was serially diluted (1 in 2) and added to appropriate tubes. To initiate the reaction, Uba6 reaction mix containing 100 nM Uba6, 25 μM ATP in 10 mM MgCl₂, 0.05% BSA, and 50 mM HEPES, pH 7.5, were added. Reaction mixtures were incubated at room temperature for 15 min before 4× LDS sample loading buffer (Invitrogen, catalog no. NP0007) was added to quench the reaction. The samples were then analyzed by SDS-PAGE under reducing conditions, transferred to 0.2-micron PVDF membrane (Millipore, catalog no. ISEQ2000), and probed with an anti-FLAG M2 antibody (Sigma). Alexa Fluor 680-labeled secondary anti-mouse antibody (Invitrogen, catalog no. A21058) was then used, and quantitation of protein bands was performed using a Li-Cor Odyssey Imaging System.

E2 HTRF® Transthiolation Assay—The HTRF® assay was run as described previously (24, 26). Biotinylated USE1 was used instead of GST-tagged. Hence, for the Uba6-USE1 reactions, 10 μg/ml streptavidin-allophycocyanin (Prozyme, Hayward, CA, catalog no. P2555) was used for detection instead.

Mass Spectrometry to Quantitate Intracellular Ubiquitin and FAT10—IM-9 cells were obtained from American Type Culture Collection (ATCC) and cultured as recommended. Cells were harvested and lysates generated using RIPA buffer (Thermo Scientific) supplemented with complete protease inhibitors (Roche Diagnostics) and 10 mM N-ethylmaleimide. Recombinant TNF-α and IFNγ were obtained from Thermo Scientific. SDS-PAGE was performed using BisTris- and MES-based systems (Invitrogen), and immunoblotting was performed using antibodies to FAT10 (Santa Cruz Biotechnology, Santa Cruz, CA, catalog no. sc-67203) and Uba6 (Millennium). 15N-Labeled FAT10 and ubiquitin internal standards were generated by expressing FAT10 or ubiquitin pDEST14 vectors into Rosetta/DE3 cells (EMD Biosciences, Inc., Darmstadt, Germany) supplemented with BioExpress cell growth media (U-15N, 98%, Cambridge Isotope Laboratories, Inc., Andover, MA). Proteins were purified using HiTrap SP column and Superdex 75 column (GE Healthcare).

Lysates were prepared from asynchronous IM-9 cells treated with or without TNF-α/IFNγ (400/200 units) for 18 h. For mass spectrometry analysis, universal 15N-labeled ubiquitin and FAT10 internal protein standards were added to the cell lysate and were mixed with 4× LDS gel loading sample buffer, followed by reduction (20 mM DTT at 60 °C for 10 min) and alkylation (100 mM N-ethylmaleimide at RT for 1 h). For analysis under nonreducing conditions, the reduction reaction was skipped. The lysates were separated on a 4 – 12% NuPAGE BisTris gel (Invitrogen). The gel bands containing free ubiquitin and FAT10 proteins were excised and subjected to in-gel tryptic digestion using a published protocol (28).

Digested protein samples were analyzed on a LC/MS/MS system comprised of an Eksigent NanoLC Ultra 2D-Plus liquid chromatography system, a NanoLC AS-2 autosampler (Eksigent, Dublin, CA), and a LTQ-Orbitrap Velos mass spectrom-
with FAT10 and only Uba6 showed detectable activity (supplemental Fig. 1B). These results suggest that FAT10 is exclusively activated by Uba6. Consistent with earlier studies (13–15), ubiquitin was activated by both Uba6 and UAE (data not shown).

Quantitative analysis of the amount AMP released during the activation process suggested that Uba6 forms a ternary complex in reactions with ubiquitin or FAT10. The mechanism proposed for UAE (Fig. 1A) predicts that 2 eq of ATP are hydrolyzed during the three-step activation cycle to generate 1 eq of AMP and UBL-adenylate (6). In this assay, the UBL adenylate intermediate is converted to AMP during the analysis (25). For Uba6, approximately 2 eq of AMP were produced in reactions with either ubiquitin or FAT10, which supports the formation of a ternary complex (Table 1). Moreover, only 1 eq of AMP was detected in the presence of the inhibitor Compound 1 or when the active site cysteine mutant (C625A) of Uba6 was tested. Together, these data are consistent with the established mechanism of inhibition of Compound 1 and the role of the Uba6-S–UBL thioester (step 2) in ternary complex formation (6, 7, 24, 25).

Note that a mutant FAT10 was used in these experiments in which each cysteine residue was changed to serine (C7S, C9S, C134S, and C162S). This was done to minimize in vitro side reactions attributed to high nucleophilicity and redox chemistry of the cysteine residues in FAT10. We found that Cys-162 of FAT10 was particularly susceptible to attacking the Uba6-S–UBL thioester bond between the C-terminal glycine of FAT10 and the catalytic cysteine of Uba6, forming an intramolecular cyclized FAT10 species (supplemental Fig. 2, A–E). This cyclized FAT10 species is no longer a substrate for Uba6, which complicates the stoichiometric and kinetic analysis of its activation by Uba6 (supplemental Table 1). Therefore, the FAT10 mutant was constructed for this study following a strategy adopted for studies of ISG15, a UBL that contains two ubiquitin-like domains with an internal reactive cysteine (29).

**RESULTS**

**Uba6 Utilizes a Three-step Mechanism to Activate Ubiquitin and FAT10**—Both ubiquitin and FAT10 have previously been shown to be substrates for Uba6 (13). In this study, Uba6 was tested using a panel of UBLs for the ability to support ATP-PP exchange (6, 7, 27). The ATP-PP exchange assay measures the UBL-dependent incorporation of radioactive PPi into ATP (6, 7, 24, 25). The ATP-PP exchange activity with ubiquitin and FAT10 exclusively (supplemental Fig. 1A). We also tested each of the eight known human E1s for the ability to catalyze ATP-PP exchange

| Samples                     | Ratio of AMP/Ub6 |
|-----------------------------|------------------|
| WT Uba6 + ubiquitin         | 2.24 ± 0.11      |
| WT Uba6 + ubiquitin + Compound 1 | 1.20 ± 0.27 |
| C625A Uba6 + ubiquitin      | 0.96 ± 0.15      |
| WT Uba6 + FAT10             | 2.02 ± 0.06      |
| C625A Uba6 + FAT10          | 0.91 ± 0.17      |
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TABLE 2
Kinetic analysis of ubiquitin and FAT10 activation by Uba6 in the ATP-PPi exchange assay

| Uba6 reaction | $K_{D}$ UBL | $K_{D}$ ATP | $K_{D}$ PPi | $k_{cat}$ |
|---------------|-------------|-------------|-------------|---------|
| Ubiquitin     | $0.56 \pm 0.020^{a}$ | $15 \pm 1.7$ | $3.8 \pm 0.18$ | $0.83 \pm 0.058$ |
| FAT10         | $0.067 \pm 0.011$ | $3.3 \pm 0.23$ | $1.0 \pm 0.19$ | $0.14 \pm 0.015$ |

$^{a}$ Inhibited top points were excluded from estimated $K_{D}$ fit for ubiquitin.

TABLE 3
Enzymatic rates of catalysis for ubiquitin and FAT10 activation by Uba6

Pre-steady state rates of adenylate and thioester formation were determined using rapid chemical quench. UBL-AMP formation was monitored using the TLC AMP release assay. Ubiquitin-S–UBL thioester formation was monitored using a Western blot-based assay. Steady state rates of USE1–UBL formation were determined by monitoring the levels of USE1-thioester over time using a Western blot-based assay and fitting to a linear regression.

| Uba6 reaction | UBL-AMP$^{a}$ | Uba6-S–UBL$^{a}$ | USE1–UBL |
|---------------|--------------|-----------------|----------|
| Ubiquitin     | $9.7 \pm 1.3$ | $1.0 \pm 0.20$  | $0.40 \pm 0.014$ |
| FAT10         | $5.9 \pm 0.53$ | $0.87 \pm 0.17$ | $0.16 \pm 0.047$ |

$^{a}$ The $K_{D}$ values for UBL-AMP and Uba6-S–UBL formation were determined by plotting quantified signal versus time and fitting to the single exponential rise equation, $Y = Y_0 \times \left(1 - \exp(-k_{cat} \times t)\right)$.

**FAT10 Binds Tighter than Ubiquitin to Uba6**—We next conducted binding studies to directly measure the affinity between Uba6 and ubiquitin or wild-type FAT10 in the absence of ATP-Mg$^{2+}$. Ubiquitin showed very weak affinity for Uba6 with a $K_{D}$ greater than 20 $\mu$M (Fig. 3A), which was significantly higher than the apparent $K_{D}$ (0.56 $\mu$M) measured in the ATP-PPi exchange assay (Table 2). These results are consistent with observations made with UAE and ubiquitin (25). However, tight 1:1 binding was observed for FAT10 with Uba6 in the absence of ATP (Fig. 3A). The $K_{D}$ value obtained for FAT10, 0.055 $\mu$M, was in a similar range as the $K_{D}$ measured in the ATP-PPi exchange assay (0.067 $\mu$M) (Table 2).

The large difference in affinities with Uba6 suggests that FAT10 could effectively compete with ubiquitin for activation. As demonstrated above, Uba6 showed 6-fold lower activity in the ATP-PPi exchange assay with FAT10 compared with ubiquitin, making it possible to design a substrate competition assay. When the concentration of ubiquitin was fixed at 1 $\mu$M and FAT10 was titrated into the reaction mixture, a clear inhibition of the ATP-PPi exchange activity was observed until the rate decreased to the level of the reaction containing FAT10 alone (Fig. 3B). The resulting dose-response curve was fitted to give an $IC_{50}$ value of 0.064 $\mu$M, consistent with both the $K_{D}$ (0.055 $\mu$M) and $K_{D}$ (0.067) measured for FAT10. We determined that neither of the FAT10 ubiquitin-like domains alone or mixed together could efficiently compete with ubiquitin, suggesting that the overall di-ubiquitin-like structure of FAT10 was important for tight binding to Uba6 (supplemental Fig. 4B).

In reactions where the concentration of FAT10 was fixed at 1 $\mu$M, ubiquitin was unable to effectively compete with FAT10 for binding to Uba6, even at concentrations up to 10 $\mu$M (Fig. 3B). These results suggest that Uba6 should be able to switch its catalytic functions between FAT10 and ubiquitin depending on their relative concentrations.

**Inhibition of Uba6 with Compound 1**—Compound 1 was previously identified as a mechanism-based inhibitor that is active...
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When FAT10 was the substrate, Uba6 was not significantly different from Uba6 and was followed by desalting to remove excess compound. The Uba6-adduct complex was diluted into the ATP-PPi exchange assay to a final concentration of 5 nM, which is well below the IC50 of Compound 1. Ubiquitin-Compound 1 adduct was found to be tightly bound to Uba6, as even after 4 h little enzymatic activity had recovered relative to the control (Fig. 4D). For FAT10, however, the activity of Uba6 completely recovered over a time range of 5–15 min. These results suggest that both the slow rate of adduct formation and weak adduct binding to Uba6 contributed to the weak potency of Compound 1 in reactions with FAT10. Consistent with this hypothesis, the purified ubiquitin-Compound 1 adduct was able to inhibit both ubiquitin and FAT10-mediated reactions with similar potencies (Fig. 4B). Attempts to isolate and test purified FAT10-Compound 1 adduct were unsuccessful due to the instability of the FAT10 protein under purification conditions.

We next assessed the relative levels of UBL-Compound 1 adducts formed in mixtures containing varying ratios of FAT10 and ubiquitin. First, the concentration of ubiquitin was fixed at 0.5 μM, and increasing concentrations of FAT10 were added to a reaction mixture containing Uba6, ATP, and Compound 1. UBL-Compound 1 adducts were then separated by SDS-PAGE and quantified by Western blot analysis using a Compound 1-specific antibody (24, 25). As shown in Fig. 5A, the ubiquitin-Compound 1 adduct signal was reduced and the FAT10-Compound 1 adduct signal increased as a function of increased FAT10 concentration. Fitting the competition curves of either the decrease in the ubiquitin-Compound 1 level (0.26 μM) or the increase in FAT10-Compound 1 level (0.16 μM) yielded similar apparent K1/2 values for FAT10. When the FAT10 concentration was fixed at 0.5 μM and increasing concentrations of ubiquitin were titrated into the reaction mixture, the levels of the ubiquitin-Compound 1 adduct increased with an apparent K1/2 of 0.14 μM (Fig. 5B). However, the decrease in the FAT10-Compound 1 level was less prominent, and the apparent K1/2 was ~7 μM, more than 25-fold higher than the value obtained for the increase of the ubiquitin-Compound 1 adduct in the same study. The discrepancy of the two apparent K1/2 values for ubiquitin suggests that the formation of ubiquitin-Compound 1 adduct is not capable of completely inhibiting the binding of FAT10 and the subsequent formation of the FAT10-Compound 1 adduct. This result is also consistent with the high affinity of FAT10 to Uba6 observed in earlier binding and competition assays.

Kinetic Analysis of E1-E2 Transthiolation Mediated by Uba6—To gain insight into Uba6-mediated UBL activation and transfer, the steady state rate of Uba6-USE1 transthiolation was investigated using Western blot-based analysis. The maximal rate of transthiolation from Uba6 to USE1 was ~2.5-fold slower with FAT10 than with ubiquitin, suggesting that the bulkier FAT10 is not transferred as efficiently as ubiquitin (Table 3). Furthermore, using a homogeneous time-resolved fluorescence (HTRF®)-based assay to measure formation of E2–UBL, ATP at higher concentrations was found to significantly inhibit Uba6-USE1 transthiolation (Fig. 6A). However, higher concentrations of ATP showed minimal inhibition of the UAE-catalyzed ubiquitin transfer to Ubc2 (Fig. 6A).
To further investigate this effect, the steady state rate of USE1-S-UBL formation was measured at low and high concentrations of ATP using Western blot-based analysis. Similar to the HTRF® assay results, the rate of USE1-S-UBL thioester formation was inhibited by high concentrations of ATP (supplemental Fig. 5, A–B). To investigate whether ATP inhibition was USE1- or Uba6-specific, the steady state rate of ubiquitin transthiolation from Uba6 to UbcH5a was examined at various concentrations of ATP. It was found that although the maximal rate of ubiquitin transfer from Uba6 to UbcH5a was 30-fold

**FIGURE 4.** Uba6 forms ubiquitin and FAT10-Compound 1 adduct but slower rate of FAT10-Compound 1 formation and weaker binding leads to reduced potency. A, Compound 1 was titrated into Uba6 ATP-PPi reactions containing either ubiquitin (●) or FAT10 (○). IC50 fits were 0.15 and >100 μM for ubiquitin and FAT10 reactions, respectively. B, purified ubiquitin-Compound 1 was titrated into Uba6 ATP-PPi reactions containing either ubiquitin (●) or FAT10 (○). IC50 values were 0.017 and 0.012 μM for ubiquitin and FAT10 reactions, respectively. C, rates of ubiquitin- and FAT10-Compound 1 (Ub-1 and FAT10–1) formation were determined in Uba6 reactions containing ATP, Compound 1, and either ubiquitin (●) or FAT10 (○). Levels of UBL-Compound 1 (UBL-1) formed were monitored using Western blot-based assay. Signal was quantified, and UBL-1 standard curve was used to determine picomoles of UBL-1 produced. Linear regression of curves was used to determine rates of 0.015 and 0.0055 pmol/s for ubiquitin and FAT10, respectively. D, reversibility of Ub-1 and FAT10–1 inhibited Uba6 was measured in the ubiquitin-catalyzed ATP-PPi exchange assay. Uba6, ATP, DMSO (●), or Compound 1, and either ubiquitin or FAT10 was incubated to form Ub-1- (●) or FAT10–1 (○)-bound Uba6. Samples were desalted and diluted 1 in 100 into a pyrophosphate exchange assay containing 1 mM ATP, and 2 μM ubiquitin and recovery was monitored over time at 30 °C.

**FIGURE 5.** FAT10 and ubiquitin compete for UBL Compound 1 adduct formation on Uba6. A, FAT10 was titrated into reactions containing Uba6, ATP, 20 μM Compound 1, and ubiquitin fixed at 0.5 μM. B, ubiquitin was titrated into reactions containing Uba6, ATP, 20 μM Compound 1, and FAT10 fixed at 0.5 μM. Top panel, Western blot image using anti-Compound 1 antibody to detect adduct. Bottom panel, plots of quantified levels of Ub-1 (●) or FAT10–1 (○) signal were fit as described to determine K1/2 values.
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**Evaluation of free ubiquitin and FAT10 levels in nonstimulated and TNF-α/IFNγ-stimulated IM-9 cells**

Lysates prepared from asynchronous IM-9 cells treated with or without TNF-α/IFNγ (400/200 units) for 18 h were analyzed using reducing or nonreducing SDS-PAGE and Coomassie stain. Mass spectrometry analysis was performed using an in-gel trypsin digest with region excised consistently by molecular weight with ubiquitin or FAT10. Several ubiquitin- and FAT10-specific tryptic peptides were identified and quantified from the 15N-labeled ubiquitin or FAT10 internal standard. The average of all peptides detected is reported here.

| Sample      | Reduced | Nonreduced |
|-------------|---------|------------|
| TNF-α/IFNγ  |         |            |
| −TNF-α/IFNγ | fmol µg⁻¹ | fmol µg⁻¹  | Reduced | Nonreduced |
| Ubiquitin   | 0.25 ± 0.02 | 3.1 ± 0.25 | 1.7 ± 0.20 |
| FAT10       | 0.34 ± 0.07 | 3.1 ± 0.25 | 1.7 ± 0.20 |

**Cellular Analysis of Ubiquitin and FAT10**—The binding and competition studies presented above suggest that FAT10 has much greater affinity to Uba6 than ubiquitin. To understand the biological relevance of this observation in a cellular context, levels of free ubiquitin and FAT10 were quantified in IM-9 cells. IM-9 cells were chosen because we found that they up-regulated expression of FAT10 following TNF-α/IFNγ stimulation. Using 15N-labeled ubiquitin and FAT10 standards, levels of free ubiquitin and free FAT10 were quantitated in IM-9 lysates prepared under nonreducing or reducing conditions using mass spectrometry. Under nonstimulated and nonreducing conditions, the average levels of free FAT10 were ~0.30 ± 0.02 fmol/µg cell lysate (Table 4). When samples were run under reducing conditions, the average levels of free FAT10 were similar, 0.34 ± 0.07 fmol/µg cell lysate. The observation of similar levels of free FAT10 in reducing and nonreducing samples suggests that under nonstimulated conditions, very little FAT10 is incorporated as a thioester (Table 4). Furthermore, under cytokine-stimulated conditions, the level of free FAT10 under nonreduced conditions increased to 1.7 ± 0.20 fmol/µg, whereas levels under reduced conditions increased to 3.1 ± 0.25 fmol/µg (Table 4). In contrast, levels of free ubiquitin in nonstimulated conditions were found be to ~55 ± 0.80 fmol/µg under nonreduced conditions and 94 ± 4.0 fmol/µg under reduced conditions. This difference in levels of “free” ubiquitin in reduced versus nonreduced conditions suggests that more than 40% of total free ubiquitin detected in the reduced samples is incorporated as a thioester (Table 4). The levels of free ubiquitin in cytokine-stimulated cells increased slightly to 64 ± 0.90 and 107 ± 5.0 fmol/µg in nonreduced and reduced conditions, respectively; however, the percentage of ubiquitin incorporated in thioesters did not change (Table 4). Overall, free FAT10 levels increased 6–9-fold under TNF-α/IFNγ-stimulated conditions, and the ratio of free ubiquitin (nonreduced) available to compete with free FAT10 went from 160-fold under nonstimulated conditions to ~20-fold under stimulated conditions. This suggests that under nonstimulated conditions, FAT10 may not be as efficiently activated by Uba6, and the Uba6 pathway may be biased toward ubiquitin conjugation. This is consistent with the results showing minimal amounts of FAT10 incorporated in thioester under nonstimulated conditions. Upon cytokine stimulation, however, the ratio of available ubiquitin and free FAT10 decreased to the extent that cellular FAT10 activation is feasible. This was reflected in a...
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FIGURE 7. Overexpression of FAT10 has minimal effect on levels of Uba6-S–ubiquitin thioester in IM-9 cells. Lysates prepared from asynchronous IM-9 and cells were treated using TNF-α/IFN-γ (400/200 units) for 18 h and were analyzed by immunoblotting with antibodies to Uba6 and FAT10. Reduction as indicated was done using 100 mm β-mercaptoethanol.

45% increase in thioester-incorporated FAT10 under stimulated conditions. Moreover, these results suggest that the relative ratio of ubiquitin and FAT10 in cells could serve as a cellular regulatory mechanism to modulate the Uba6 pathway. Interestingly, increased expression of FAT10 following stimulation showed a minimal effect on the Uba6-S–ubiquitin level in IM-9 cells (Fig. 7), suggesting that the up-regulation of the FAT10 conjugation pathway is not accompanied by suppression of ubiquitin activation. In addition, Uba6-S–FAT10 thioester was not detected in stimulated IM-9 cells (data not shown), suggesting that FAT10 activation and transfer catalyzed by Uba6 might be extremely transient.

DISCUSSION

UAE and other E1s activate their cognate UBLs through a conserved three-step catalytic mechanism that involves formation of a UBL adenylate and thioester intermediate (1, 6, 30). In this study, we characterized Uba6 and found that Uba6 follows a similar three-step mechanism. Stoichiometric analysis of AMP production in a single turnover setting with wild-type and catalytically inactive Uba6 (C625A) was consistent with the formation of a ternary complex (Table 1). Similar to NAE and NEDD8 (30), the mechanism of ubiquitin binding to Uba6 was pseudo-ordered; however, the titration profile for FAT10 was consistent with random binding (Fig. 2). Although low solubility of FAT10 prevented us from testing at higher concentrations, titration studies in the ATP-PPi exchange assay along with binding studies using SPR suggest that the mechanism of binding of FAT10 and ATP to Uba6 may be formally random. Taken together, these results suggest that Uba6 binds ubiquitin and FAT10 through different binding mechanisms.

Although ubiquitin and FAT10 may have different mechanisms of binding, the rates of Uba6-catalyzed ubiquitin- and FAT10-adenylate and thioester formation were similar (Table 3). This was somewhat surprising considering the large difference in size and structure of ubiquitin and FAT10 and the large conformational change required for precise alignment of the catalytic cysteine on the E1 and the C-terminal glycine on the UBL during thioester formation (31–34). However, we did find that rates for FAT10-dependent Uba6 ATP-PPi exchange were significantly slower than ubiquitin-dependent ATP-PPi exchange (Table 2).

Multiple experiments confirmed that Uba6 bound FAT10 with significantly higher affinity than ubiquitin (Figs. 3 and 5). Based on the high affinity of Uba6 for FAT10, one could consider that each ubiquitin-like domain of FAT10 individually contributes to binding and affinity. However, when each of the ubiquitin-like pieces of FAT10 were made separately, N-terminal amino acids 1–81 and C-terminal amino acids 90–165, it was found that neither piece was capable of inhibiting the ubiquitin reaction individually or mixed together (supplemental Fig. 4). Also, the N-terminal truncated pieces with or without GG substituted for VV and C-terminal truncated pieces of FAT10 whether alone or mixed together had no detectable pyrophosphate exchange activity and could not form Uba6 thioester suggesting that these individual FAT10 pieces bind weakly to Uba6 (data not shown). Furthermore, when FAT10 constructs were made in which the two C-terminal glycines were deleted (ΔGG FAT10), it was found that this truncated FAT10 could still inhibit ubiquitin-dependent Uba6 ATP-PPi exchange, albeit at a reduced affinity compared with wild-type FAT10 (0.32 versus 0.064 μM) (supplemental Fig. 4B). Conversely, neither full-length nor ΔGG ubiquitin was effective at competing with the FAT10-dependent reaction (Fig. 3B and data not shown). Also, neither the ΔGG FAT10 nor the ΔGG ubiquitin was capable of inhibiting the pyrophosphate-catalyzed reactions of their respective full-length versions (data not shown). This indicates that FAT10 surface interactions other than the C terminus contribute significantly to the affinity for Uba6. Taken together, these studies reveal a remarkable feature of the Uba6 active site to engage a single ubiquitin and a di-ubiquitin like FAT10 but also to reject the individual UBL domains of FAT10 if not structured in the proper orientation.

Previous studies confirmed that Compound 1 is a mechanism-based inhibitor of several E1 enzymes, including Uba6 (24). In this study, we confirmed that Uba6 forms a Compound 1 adduct with both ubiquitin and FAT10 (Fig. 5). Compound 1 was found to be a potent inhibitor of the ubiquitin-catalyzed Uba6 reaction but a poor inhibitor of the FAT10-catalyzed reaction (Fig. 4A). Follow up studies found that a slower rate of FAT10-Compound 1 adduct formation and weaker binding affinity of FAT10-Compound 1 contributed to weaker potency (Fig. 4, B and C). Competition and binding studies found that FAT10 had a higher affinity for Uba6 than ubiquitin. However, reversibility studies showed that FAT10-Compound 1 adduct was a weak binder of Uba6, whereas ubiquitin-Compound 1 adduct was a tight binder of Uba6 (Fig. 4D). This contradicts much of the competition data that consistently showed that FAT10 binds more tightly than ubiquitin to Uba6. It has been shown for NAE and UAE that the affinity for NEDD8 and ubiquitin increases several orders of magnitude when there is adenine-like sulfamate adduct on the C-terminal glycine of the UBL (24, 25). It could be that with FAT10, the addition of Compound 1 at the C terminus has minimal effect at improving its already strong affinity. In the reversibility experiment, after dilution into the pyrophosphate exchange assay, the concentration of Uba6 and presumably bound FAT10-Compound 1 adduct was 5 μM, whereas the concentration of ubiquitin was 2
μM. Competition studies in the pyrophosphate exchange assay show that in the presence of 1 μM ubiquitin and 10 nM FAT10, ubiquitin was able to outcompete FAT10 for binding (Fig. 3B). Altogether, this suggests that unlike with ubiquitin, the formation of a FAT10-Compound 1 adduct does not result in an improved affinity for Uba6.

UAE and Uba6 were found to differentially charge E2 enzymes with ubiquitin (14). Some E2s were found to be charged by both E1s, and others were found to be only charged by UAE; and one E2, USE1, was found to be exclusively charged by Uba6 (14). More recently, Aichem et al. (18) found that Uba6 is also capable of charging USE1 with FAT10. Consistent with these reports, we found that USE1 was able to be charged with ubiquitin and FAT10, although the rate of ubiquitin charging was 2.5-fold faster than FAT10 (Table 3). Although Uba6 was found to be able to charge UbcH5a with ubiquitin, the rate was almost 30-fold slower than that of USE1, suggesting that UbcH5a may not be a true biological E2 for Uba6 (supplemental Fig. 5C). Interestingly, it was found that high concentrations of ATP inhibited UBL transthiolation from Uba6 to USE1 (Fig. 6A and supplemental Fig. 5, A–C). This phenomenon was shown to be Uba6-specific as transthiolation to UbcH5a was also inhibited at high concentrations of ATP, whereas transthiolation of ubiquitin from UAE to Ubc2 was minimally affected (Fig. 6A and supplemental Fig. 5C). Studies using C188A USE1 suggest that the effect of ATP is not a binding phenomenon between UAE and USE1 but most likely is attributed to the catalytic mechanism of transthiolation from Uba6 to E2 (supplemental Fig. 5, D and E). It appears that during the Uba6–E2 catalytic reaction, the formation of Uba6–ubiquitin thioester becomes the rate-limiting step at high concentrations of ATP. This is emphasized by the significant reduction of Uba6–S-ubiquitin in the presence of wild-type USE1 and the incremental reduction in USE1–UBL thioester that was found at increasing Uba6 turnover at high concentrations of ATP (Fig. 6B and data not shown). It has been found that an E1–E2 transthiolestion event requires significant conformational rearrangements of the E1 enzyme (31, 34, 35). Crystallography studies with the NAE ternary complex and Ubc12 show that the core domain of Ubc12 associates with the UFD of NAE and a peptide-like extension on Ubc12 docks into a groove in the adenylation domain, adjacent to the ATP-binding site on NAE (35). It is hypothesized that formation of NEDD8-adenylate on the NAE–NEDD8 thioester induces a rotation in the UFD domain of almost 120°, which enables NAE to bind Ubc12 and align it in prime position for transthiolation (35). After the NEDD8 thioester is transferred to Ubc12, the loss of the covalent bond with NEDD8 induces the UFD of NAE to rotate back (35). Considering the mechanism proposed for NAE, we hypothesize that high concentrations of ATP could trap the UFD domain of Uba6 in a confirmation that is unfavorable for UBL charging. Similarly, we wonder whether a cryptic E2 binding groove near the ATP binding pocket of Uba6 may also play a role. More studies into the interaction of Uba6 and USE1 at high concentrations of ATP are necessary for further understanding the mechanism of ATP inhibition.

At any given time, a large pool of ubiquitin is available in a cell; however, a vast majority of this ubiquitin is attached onto proteins. Kaiser et al. (36) have shown that in HEK293 cells roughly 68% of all ubiquitin is mono-ubiquitinated onto a protein; 8.5% is incorporated into chains, and 23% is free ubiquitin. The “free ubiquitin” levels described are most likely an overestimation because the data were obtained under reducing conditions and so ubiquitin that is covalently attached as a thioester to E1s, E2s, and E3s within a cell would be counted as free ubiquitin. Siepmann et al. (37) found that most of the free ubiquitin in cells is incorporated as thioesters to components of conjugation pathways. In terms of competing with FAT10 for charging, it is the available free ubiquitin that is not incorporated as thioester that directly competes with FAT10 for binding to Uba6. Single turnover rates and K<sub>D</sub> values determined using Biacore suggest that Uba6 has greater than 300-fold specificity for FAT10 over ubiquitin (Table 3 and Fig. 8A). Consequently, one could speculate that in cells, Uba6 is indeed specific for FAT10 but is capable of utilizing the lower affinity ubiquitin due to its high abundance. Quantification of free ubiquitin and free FAT10 via mass spectrometry found that the ratio of available free ubiquitin (nonreduced) to free FAT10 changes from 160-fold under nonstimulated conditions to 20-fold under stimulated conditions (Table 4). Based on <i>in vitro</i> kinetic and binding studies, Uba6 should be able to efficiently charge FAT10 in the presence of 20-fold excess ubiquitin. Although we were unable to directly detect Uba6–S–FAT10 thioester in stimulated cells, the apparent increase of FAT10 thioesters in cytokine-stimulated cells (nonreduced versus reduced samples, see Table 4) suggests that increases in levels of free FAT10 upon TNF-α/IFN-γ stimulation could serve as a regulatory mechanism that strictly turns on Uba6-dependent FAT10 conjugation.

The studies described here represent the first detailed characterization of Uba6 enzymology. Uba6 follows a three-step enzymatic process similar to other canonical E1s, although it is unique in its ability to efficiently charge two highly distinct UBLs. The kinetic and binding studies comparing ubiquitin and FAT10 reveal a potential mechanism for how Uba6-catalyzed FAT10 and ubiquitin conjugation is regulated in cells. More extensive biochemical and cellular studies investigating the interaction of Uba6 with FAT10 and ubiquitin are required to further the understanding of the dynamics of the Uba6 conjugation pathways.

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