Structural Determinants of Ubiquitin Conjugation in Entamoeba histolytica*

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Background: Ubiquitination plays critical roles in many cellular processes.

Results: The Entamoeba histolytica ubiquitin activating, conjugating, and ligating enzymes interact and transfer ubiquitin.

Conclusion: E. histolytica possesses a functional ubiquitination cascade with key differences from mammalian homologs.

Significance: The E. histolytica ubiquitin-proteasome pathway may provide therapeutic targets for amoebic colitis and amoebiasis.

Ubiquitination is important for numerous cellular processes in most eukaryotic organisms, including cellular proliferation, development, and protein turnover by the proteasome. The intestinal parasite Entamoeba histolytica harbors an extensive ubiquitin-proteasome system. Proteasome inhibitors are known to impair parasite proliferation and encystation, suggesting the ubiquitin-proteasome pathway as a viable therapeutic target. However, no functional studies of the E. histolytica ubiquitination enzymes have yet emerged. Here, we have cloned and characterized multiple E. histolytica ubiquitination components, spanning ubiquitin and its activating (E1), conjugating (E2), and ligating (E3) enzymes. Crystal structures of EhUbiquitin reveal a clustering of unique residues on the ε1 helix surface, including an eighth surface lysine not found in other organisms, which may allow for a unique polyubiquitin linkage in E. histolytica. EhUbiquitin is activated by and forms a thioester bond with EhUba1 (E1) in vitro, in an ATP- and magnesium-dependent fashion. EhUba1 exhibits a greater maximal initial velocity of pyrophosphate:ATP exchange than its human homolog, suggesting different kinetics of ubiquitin activation in E. histolytica. EhUba1 engages the E2 enzyme EhUbc5 through its ubiquitin-fold domain to transfer the EhUbiquitin thioester. However, EhUbc5 has a >10-fold preference for EhUba1-Ub compared with unconjugated EhUba1. A crystal structure of EhUbc5 allowed prediction of a noncovalent “backside” interaction with EhUbiquitin and E3 enzymes. EhUbc5 selectively engages EhRING1 (E3) to the exclusion of two HECT family E3 ligases, and mutagenesis indicates a conserved mode of E2/RING-E3 interaction in E. histolytica.

Note: ULM, ubiquitin-like modifier; UFD, ubiquitin-fold domain; HECT, homology to the E6AP C terminus; NTA, nitrilotriacetic acid; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; SPR, surface plasmon resonance; PDB, Protein Data Bank; C.I., confidence interval.

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Crystal Structures of EhUbiquitin and EhUbc5

with the latter characterized by cysts in liver, lungs, and brain (10). Spread in an encysted form, E. histolytica infection is endemic in developing countries with poor barriers between sewage and drinking water (11). Although E. histolytica has been the subject of research for more than 50 years, the relatively recent sequencing of its genome (12) affords the opportunity for further insight into cellular machinery that may be amenable to pharmacologic manipulation, such as the ubiquitin-proteasome pathway. The cloning and characterization of an E. histolytica ubiquitin gene (termed EhUbiquitin) highlighted a surprising degree of sequence divergence from homologs in humans and lower organisms, given the remarkably high degree of conservation among other species (13). Despite its relatively dissimilar sequence, EhUbiquitin complements deletion of the polyubiquitin gene ubi4 in yeast, suggesting conserved functions in E. histolytica (14). More recent bioinformatic analyses of the E. histolytica genome revealed an extensive family of putative ubiquitin activating, conjugating, and ligating enzymes, as well as parallel systems for other ubiquitin-like modifiers (15). However, functional studies of this putative ubiquitination machinery have not yet emerged. Interestingly, treatment with proteasome inhibitors impairs growth of E. histolytica trophozoites and encystation in the related species Entamoeba invadens, suggesting that the ubiquitin–proteasome pathway may be a valuable therapeutic target (16). Altered expression of ubiquitin-proteasomal genes has also been correlated with perturbations in virulence (e.g. Ref. 17). Our own study of heterotrimeric G-protein signaling in E. histolytica demonstrated that markers of trophozoite virulence are enhanced or reduced upon overexpression of the Go subunit, EhGα1, or a dominant-negative EhGα1 mutant, respectively (18). A transcriptome analysis by RNA-seq revealed differential expression of multiple ubiquitin-proteasome pathway-related genes upon expression of wild-type or mutant EhGα1, including the Ehubiquitin gene itself (Table 1). In the present study, we sought to characterize, both structurally and biochemically, various components of the E. histolytica ubiquitination machinery, spanning ubiquitin and its interacting E1–E3 enzymes. We hypothesize that differences revealed between the E. histolytica components and well studied mammalian homologs may elucidate a potential advantage for specific targeting of ubiquitination within the parasitic amoeba.

**EXPERIMENTAL PROCEDURES**

**Cloning and Protein Purification—**Genomic DNA was isolated from the virulent HM-1:IMSS strain of E. histolytica using a DNeasy Blood and Tissue Kit (Qiagen). Open reading frames of EhUbiquitin (AmoebaDB accession EHI_083410), Ehubc5 (EHI_020270), Ehuc5 (EHI_038560), Ehring1 (EHI_020100), Ehhect1 (EHI_011530), and Ehhect2 (EHI_124600) were PCR amplified from genomic DNA and subcloned as hexahistidine fusions into a pET vector-based ligation-independent cloning vector, pLIC-His, as described previously (19). PCR primer sequences were: Ehubiquitin, 5’-ATGCAAATATTGTTAAGAC-3’ and 5’-TTAGTGATAAATAATTCGGTG-3’; Ehubc5, 5’-ATGACAAAACAAATTGAGACGCGTGATG-3’ and 5’-TTAGAAATCAGAACAATCAGCTGGAAATT-3’; Ehuc5, 5’-ATGGGTATGCGTAGAATTCAAAAAG-3’ and 5’-TTAGAAATCAGAACAATCAGCTGGAAATT-3’; Ehring1, 5’-ATGTCAAGAGAATAAACAATGACACAAACAAATGGTAAGACGCGTGATG-3’ and 5’-TTAGAAATCAGAACAATCAGCTGGAAATT-3’; Ehhect1, 5’-ATGACAAAACAAATTGAGACGCGTGATG-3’ and 5’-TTAGAAATCAGAACAATCAGCTGGAAATT-3’. Fragments subcloned and purified as recombinant proteins included the ubiquitin-fold domain of EhUb1 (amino acids 1–246), EhHect1 HECT domain (amino acids 277–660), and EhHect2 HECT domain (amino acids 882–984). Point mutations to Ehubc5 were made using PCR and the overlap extension method (20). Recombinant human Uba1, derived from insect cells, was purchased from Boston Biochem. Ubiquitin from bovine eryth-

| Table 1 | Ubiquitin and proteasome system genes differentially transcribed in E. histolytica trophozoites expressing EhGα1 or the dominant-negative EhGα1S37C expression. | | Fold-change upon EhGα1 expression | Fold-change upon EhGα1S37C expression |
|---|---|---|---|---|
| Gene name | AmoebaDB accession no. | p value | | |
| Ubiquitin system/proteasome | | | | |
| Hypothetical (RING finger domain) | EHI_0104520 | 1.9 (0.004) | | |
| Ubiquitin-activating enzyme | EHI_008450 | 1.7 (0.013) | | |
| Zinc finger protein (RING-type) | EHI_165120 | 1.8 (0.004) | | |
| Ub1 protease family, C-terminal catalytic | EHI_138530 | 2.2 (0.002) | | |
| F-box domain containing protein | EHI_103710 | −1.9 (0.007) | | |
| 26 S Protease regulatory subunit | EHI_185410 | −1.8 (0.011) | | |
| RING zinc finger protein | EHI_023510 | −2.1 (0.008) | | |
| Ubiquitin (RING-type) | EHI_027910 | −2.5 (<0.001) | | |
| Proteasome subunit a type 3 | EHI_098060 | −2.0 (0.007) | | |
| Zinc finger protein (RING-type) | EHI_130650 | −1.9 (0.004) | | |
| Zinc finger, RING-type | EHI_159840 | −2.1 (<0.001) | | |
| Proteasome subunit a type 2-A | EHI_052140 | 1.7 (0.019) | | |
| WWE domain | EHI_069610 | 1.7 (<0.001) | | |
| Ubiquitin | EHI_083410 | 2.2 (0.001) | | |
| Zinc finger, RING-type | EHI_110790 | 2.3 (<0.001) | | |
| Ubiquitin-conjugating enzyme E2 | EHI_131530 | 2.0 (<0.001) | | |
| Ubiquitin-conjugating enzyme E2 | EHI_135460 | 1.8 (0.009) | | |
| 26 S Protease regulatory subunit | EHI_177520 | 1.9 (<0.001) | | |
| 19 S Cap proteasome S2 subunit | EHI_198010 | 3.6 (<0.001) | | |
| 26 S Protease regulatory subunit | EHI_053020 | 2.2 (0.020) | | |
rocytes was purchased from Sigma. Because the sequences of bovine and human ubiquitin are identical, the protein is referred to as human ubiquitin throughout the study, for clarity. For each of the E. histolytica components, BL21 Escherichia coli were grown to an \( A_{600\text{nm}} \) of 0.8 at 37 °C and expression was induced with 500 \( \mu \)M isopropyl \( \beta\)-d-thiogalactopyranoside for 14–16 h at 20 °C. Pelleted bacterial cells were resuspended in N1 buffer containing 30 mM HEPES, pH 8.0, 250 mM NaCl, and 30 mM imidazole and lysed by high-pressure homogenization with an Emulsiflex (Avestin; Ottawa, Canada). Cellular lysates were cleared by centrifugation at 100,000 \( g \) for 1 h at 4 °C, and the supernatant was applied to a nickel-nitrilotriacetic acid (NTA) FPLC column (GE Healthcare), washed extensively with N1, and eluted in N1 buffer with 300 mM imidazole. For proteins used in biochemical experiments, eluted protein was pooled and resolved using a size exclusion column (HiLoad 16/60 Superdex 200, GE Healthcare) in S200 buffer containing 50 mM HEPES, pH 7.5, and 100 mM NaCl (5 mM ZnCl\(_2\) was included in the case of EhRING1 purification). For proteins used in crystallographic studies, protein eluted from the NTA column was pooled and dialyzed into imidazole-free N1 supplemented with 5 mM DTT overnight at 4 °C in the presence of HiS\(_6\)-tobacco etch virus protease to cleave the N-terminal affinity tag. The dialysate was then passed over a second NTA column to remove tobacco etch virus protease and uncleaved protein, followed by resolution by size exclusion in S200 buffer. All proteins except EhUba1 were concentrated to 0.25–2 mM and snap frozen in a dry ice/ethanol bath for storage at −80 °C. EhUba1 was found to precipitate upon freeze/thaw, but could be stably maintained at 4 °C for at least 2 weeks. Protein concentration was determined by \( A_{280} \) nm measurements upon denaturation in 8 M guanidine hydrochloride, based on predicted extinction coefficients for each protein using Expy.

Crystal Structures of EhUbiquitin and EhUbc5—Cysts of EhUbiquitin were obtained by vapor diffusion from hanging drops at 18 °C in two different crystal forms. EhUbiquitin crystal form 1 was obtained by mixing EhUbiquitin at 17 mg/ml (1:1) with crystallization solution containing 25% (v/v) PEG 3350 and 100 mM citric acid, pH 3.5. A single crystal grew to \( 500 \times 400 \times 400 \mu M \) over 5 days, exhibiting the symmetry of space group P3\(_2\)1\(_1\) (\( a = 49.8 \AA, b = 49.6 \AA, c = 63.5 \AA, \alpha = \beta = \gamma = 90^\circ \)) and containing one monomer in the asymmetric unit. For the second crystal form, EhUbiquitin at 13 mg/ml in S200 buffer was mixed 1:1 with (and equilibrated against) crystallization solution containing 22% (v/v) PEG 3350, 200 mM LiSO\(_4\), and 100 mM BisTris, pH 5.5. Crystals grew to \( 200 \times 100 \times 100 \mu M \) over 3 days, exhibiting the symmetry of space group P2\(_1\)2\(_1\)2\(_1\) (\( a = 38.6 \AA, b = 49.9 \AA, c = 76.8 \AA, \alpha = \beta = \gamma = 90^\circ \)) and containing two monomers in the asymmetric unit. For data collection at 100 K, crystals were transferred for \( 1 \) min into crystallization solution supplemented with 25% glycerol and plunged into liquid nitrogen. A native data set was collected at the SER-CAT 22BM beamline at the Advanced Photon Source (Argonne National Laboratory). Data processing and refinement were carried out similarly to EhUbiquitin, as mentioned above. A molecular replacement solution was obtained using the crystal structure model of human UbcH5b (PDB code 2ESK), modified to exclude water. The current model contains one EhUbc5 monomer with a cobalt ion coordinated by surface residues and by a monomer from the neighboring asymmetric unit. All EhUbiquitin residues could be located in the electron density. Ramachandran plot analysis indicated 98.6% favored residues. The current model of crystal form 2 contains two EhUbiquitin monomers; residues 75–77 of chain A and 74–77 of chain B could not be accurately modeled in the electron density. Ramachandran plot analysis indicated 98.6% favored, 1.4% allowed, and 0% disallowed residues.

Crystals of EhUbc5 were obtained by vapor diffusion from hanging drops at 18 °C. EhUbc5 at 8 mg/ml in S200 buffer was mixed 1:1 with (and equilibrated against) crystallization solution containing 100 mM Tris, pH 7.5, 14% (v/w) polyvinylpyrrolidone K15, and 500 \( \mu \)M CoCl\(_2\). Hexagonal crystals grew to \( 400 \times 300 \times 200 \mu M \) over 5 days and exhibited the symmetry of space group P2\(_1\)2\(_1\)2\(_1\) (\( a = 47.0 \AA, b = 49.6 \AA, c = 63.5 \AA, \alpha = \beta = \gamma = 90^\circ \)) with one monomer in the asymmetric unit. For data collection at 100 K, crystals were transferred for \( 1 \) min into crystallization solution supplemented with 25% glycerol and plunge into liquid nitrogen. A native data set was collected at the SER-CAT 22BM beamline at the Advanced Photon Source (Argonne National Laboratory). Data processing and refinement were conducted essentially as previously described for EhUbiquitin, for clarity. For each of EhUbc5 residues and by a monomer from the neighboring asymmetric unit. All EhUbiquitin residues could be located in the electron density. Ramachandran plot analysis indicated 97.3% favored, 2.7% allowed, and 0% disallowed residues.

In Vitro Ubiquitin Transfer Assay—Ubiquitin transfer experiments were conducted essentially as previously described for NEDD8ylation (25), with adaptations to allow visualization of Ub\(_1\)-EhUb Ubiquitin transfer. Briefly, 1 \( \mu \)M EhUba1, 5 \( \mu \)M wild-type or mutant EhUbc5, and 15 \( \mu \)M EhUbiquitin were incubated at 37 °C in reaction buffer containing 50 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM MgCl\(_2\), and 5 mM ATP. The reactions were halted by denaturation in 5× nonreducing SDS sample buffer, and 50 mM DTT was added to specified reactions for 10 min prior to protein separation by denaturing SDS-PAGE and staining with Coomassie Blue.

In Vitro Polyubiquitin Chain Formation Assay—Polyubiquitin chain formation experiments were conducted essentially as previously described (26). 50 \( \mu \)M EhUba1, 1 \( \mu \)M EhUbc5, 8 \( \mu \)M N-terminal FLAG epitope-tagged EhUbiquitin, and 10 \( \mu \)M EhRING1 were incubated at 37 °C for 45 min in reaction buffer containing 50 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM MgCl\(_2\), and 5 mM ATP. Reactions were halted by denaturation in 5× reducing SDS sample buffer, and 50 mM DTT was added to all reactions 10 min prior to protein separation by SDS-PAGE. Proteins were transferred to a nitrocellulose membrane and probed with anti-FLAG (M2 monoclonal; Sigma).

PP\(_2\)ATP Radioisotope Exchange Assay—Isootope exchange assays were conducted as previously described (4). All assays
were performed at 37 °C and contained 6 nM E1 ubiquitin-activating enzyme, 1 μM ubiquitin protein, 100 μM AMP, 10 mM MgCl₂, 100 μM nonradioactive pyrophosphate (PPᵢ), and variable ATP concentrations. Reactions were initiated by addition of the E1 enzyme. Concentrations of EhUba1 and human Uba1 were determined by the Bradford method. Incubation times were <15 min and within the linear portion of the progress curve (prior to approaching equilibrium). Incorporation of ³²PPᵢ into ATP was determined by adsorbing the ATP to activated charcoal. Reactions were quenched with 5% (w/v) trichloroacetic acid (TCA) containing 4 mM nonradioactive PPᵢ, and vari-
al l volumes at increasing

### RESULTS

**Structural Features of a Divergent Ubiquitin from *E. histolytica***—To give spatial context to the variant residues within *E. histolytica* ubiquitin (13) and their effects, if any, on the overall structure, we crystallized EhUbiquitin and determined its structure in two crystal forms. Under the first set of conditions, a single crystal was obtained that yielded diffraction data extending to 1.35-Å resolution. Although the diffraction data were of otherwise high quality (Table 2), detector overload resulted in exclusion of a significant fraction of low-resolution data during processing. Incomplete low-resolution data were manifested as unusually high R-factors and average B-factors during refinement (Table 2); however, the electron density was of good quality (Fig. 1B), with clear electron density obtained for the divergent residues of EhUbiquitin upon phasing by molecular replacement with human ubiquitin (PDB code 1UBQ). We also obtained EhUbiquitin crystals under a second
condition, and a structure was determined using diffraction data to 2.15 Å (Table 2 and Fig. 1C). The data collection and refinement statistics corresponding to the second EhUbiquitin crystal form were near the mean of other deposited structures of similar resolution (22). Although the structures derived from both diffraction data sets were essentially identical, the second crystal form was utilized for further analyses due to the unusually high R-factors of crystal form 1.

Crystal Structures of EhUbiquitin and EhUbc5

A Lys-54

β3

β1

β2

HsUbiquitin

EhUbiquitin: identical

EhUbiquitin: similar

EhUbiquitin: unique

Lys-54

B

D24

P38

K27

P37

Q31

G35

S22

I26

A25

E32

C

D

β1

β2

α1

β3

β4

EhUbiquitin

MQIFKTLGTKITLEVEPN

H.s. Ubiquitin

MQIFKTLGTKITLEVEPS

D.m. Ubiquitin

MQIFKTLGTKITLEVEPS

S.c. Ubiquitin

MQIFKTLGTKITLEVEPS

D.d. Ubiquitin

MQIFKTLGTKITLEVEPS

M.m. Ubiquitin

MQIFKTLGTKITLEVEPS
The Cα trace of EhUbiquitin is highly similar to human ubiquitin (root mean square deviation 0.78 Å; Fig. 1A). This finding is consistent with the protein core residues being identical between the two homologs (13), with the exception of position 26, which exhibits a subtle variation of isoleucine in E. histolytica compared with the conserved valine in a broad diversity of other species (Fig. 1D). Mapping the variant EhUbiquitin residues compared with its human homolog revealed clustering of divergent residues on a single surface, dominated by the first α-helix and including proximal portions of the β2-α1 and β3-β4 loops (Fig. 1A). Notably, one of the residues unique to E. histolytica (Fig. 1D) is an extra surface lysine (Lys-54). Because each

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surface lysine on mammalian ubiquitin can be utilized for polyubiquitin chain formation (9), it may be that *E. histolytica* possesses a unique Lys-54 linkage polyubiquitination pattern. To identify potential interactions that may involve the divergent EhUbiquitin surface, we superimposed the EhUbiquitin structure on a number of ubiquitin complex structures available in the RCSB database. Most of the surface of ubiquitin is utilized by one or more ubiquitin-binding proteins; however, the well characterized interactions with E2 conjugating enzymes (e.g. UbcH5), HECT family E3 ligases, deubiquitinating enzymes (e.g. the SAGA complex), and ubiquitin-interacting motifs (e.g. RAP80) are not predicted to form significant interactions with the unique ubiquitin residues of *E. histolytica* (Fig. 2A). Thus, at this time, we do not predict that the sequence variation seen in EhUbiquitin will significantly affect its interactions with similar ubiquitin-binding proteins encoded by the *E. histolytica* genome. The distinct EhUbiquitin surface may be utilized for other interactions unique to *E. histolytica*. However, no specific function of the divergent EhUbiquitin surface can be ascribed at this time.

**EhUbiquitin Is Activated by the E1 Enzyme EhUba1**—The *E. histolytica* genome encodes a single predicted ubiquitin-activating E1 enzyme, as well as predicted activating enzymes for Nedd8, SUMO, and other ubiquitin-like modifiers (15). The putative ubiquitin-activating enzyme EhUba1 (AmoebaDB accession EHI_020270) was cloned from genomic DNA, expressed, and purified from *E. coli*. EhUba1 possesses a predicted domain structure similar to *Saccharomyces cerevisiae* Uba1 and other eukaryotic E1s (not shown). The sequence of EhUba1 was most similar to that of the slime mold *D. discoideum*, followed by *S. cerevisiae*, when compared with a broad spectrum of E1 enzyme sequences from different species (Fig. 3D).

**FIGURE 3.** The ubiquitin-activating enzyme EhUba1 catalyzes EhUbiquitin thioester formation and transfer to the E2 enzyme EhUbc5. A, recombinant EhUba1 derived from *E. coli* forms a thioester bond with EhUbiquitin in an ATP- and magnesium-dependent fashion, as illustrated by SDS-PAGE and Coomassie Blue staining under nonreducing conditions. Activated EhUbiquitin is seen to be transferred to the ubiquitin-conjugating enzyme EhUbc5. Each of the covalent interactions detected was sensitive to 50 mM DTT reducing agent. The loss-of-E3-binding mutant EhUbiquitin(F62A) did not significantly affect ubiquitin thioester formation. B, EhUba1 and EhUbcs were sufficient to catalyze polyubiquitin chain formation in the presence of ATP and magnesium, as detected by SDS-PAGE and Western blotting (IB) under reducing conditions. EhUba1 and EhUbcs were observed to efficiently promote formation of chains up to four ubiquitin molecules under these conditions, and addition of EhRING1 had modest effects. C, PP, ATP radioisotope exchange was utilized to compare *in vitro* ubiquitin activation by EhUba1 and human Uba1 in the presence of 6 nM E1 enzyme, 1 μM ubiquitin, 1 mM ATP, 100 μM AMP, and 100 μM nonradioactive PP. Under these conditions, EH1ba1 exhibited a statistically significant 3.5-fold increase in isotope exchange rate compared with its human homolog (460 ± 33 (95% C.I.) versus 132 ± 46 (95% C.I.) pmol/min). The observed difference in ubiquitin activation kinetics is apparently intrinsic to the E1 enzyme, because the isotope exchange rate was independent of the ubiquitin species utilized. These data represent average values of triplicate experiments with mean ± S.E. * indicates a statistically significant difference between EhUba1 and human Uba1 determined by Student’s t test. D, the dependence of human Uba1- and EhUba1-mediated isotope exchange on ATP was assessed under identical conditions as panel C, excepting ATP concentration. A Lineweaver-Burk plot allowed estimation of *Km* and *Vmax* values describing E1 activity with respect to ATP. Under these conditions, the apparent *Km* and *Vmax* values were 45 μM and 459 pmol/min (95% C.I. 392–552 pmol/min) for the *E. histolytica* components and 50 μM and 115 pmol/min (95% C.I. 93–152 pmol/min) for the human components, with respect to ATP. Each initial velocity measurement was conducted in duplicate, and the displayed results are representative of two independent experiments.
To determine whether EhUba1 was capable of activating EhUbiquitin, we examined in vitro ubiquitin transfer with purified components. EhUba1 was seen to form a typical thioester bond with EhUbiquitin that was sensitive to reduction by DTT (Fig. 3A). Magnesium and ATP were required for ubiquitin activation and thioester bond formation, suggesting a conserved mechanism of ubiquitin activation in *E. histolytica*. A number of mammalian E2 enzymes, together with an E1, are capable of catalyzing formation of isopeptide-linked polyubiquitin chains (29). In vitro polyubiquitination experiments revealed that EhUbc5, together with EhUba1 could efficiently promote formation of EhUbiquitin chains up to four molecules in length (Fig. 3B). Polyubiquitin chain formation required E2 enzyme, as well as the EhUba1 cofactors of magnesium and ATP.

We next sought to compare the activities of ubiquitin-activating enzymes from *E. histolytica* and humans. To quantify the kinetics of enzyme-dependent incorporation of pyrophosphate (PPi) into ATP, an in vitro radioactive 32PPi:ATP isotope exchange assay was employed as described previously (4). In the presence of 1 μM ubiquitin protein, 100 μM AMP, 10 mM MgCl₂, and 100 μM nonradioactive PPi, the initial rate of PPi:ATP exchange catalyzed by human Uba1 was 132 pmol/min (linear regression 95% confidence interval (C.I.) 88–176 pmol/min) (Fig. 3C), in good agreement with previous measurements.

**FIGURE 4.** EhUba1 interacts directly with EhUbc5 through its ubiquitin-fold domain, and affinity is enhanced by the presence of activated EhUbiquitin. **A,** the protein sequence of EhUbC5 was aligned to its closest homologs in yeast and humans, as well as human Ubc12. The secondary structure elements reflect the crystal structure of EhUbC5. Residues involved in interaction with E2 binding partners were derived from previously published structures (PDB IDs in parentheses). The majority of predicted interaction residues are conserved in EhUbC5. **B,** surface plasmon resonance was utilized to measure direct binding of either unconjugated EhUba1 or its isolated UFD to immobilized EhUbC5. EhUba1 binds EhUbC5 with low affinity in the absence of activated EhUbiquitin, and the UFD of EhUba1 is sufficient for binding. **C,** EhUba1 was allowed to form covalent linkage with activated EhUbiquitin prior to injection over immobilized EhUbC5. Residual resonance following injection of EhUba1-EhUb was sensitive to the reducing agent DTT, likely indicating thioester transfer of EhUbiquitin to the immobilized EhUbC5. **D,** the apparent affinity of EhUba1-EhUb for EhUbC5 was significantly higher than that of unconjugated EhUba1 (panel B). The apparent KD value of ~12 μM is likely overestimated because less than 100% of the injected EhUba1 is expected to be conjugated to EhUbiquitin (Fig. 3) and equilibrium of binding could not be reached under these experimental conditions (panel C). The loss of E3-binding mutant EhUbC5(F62A) did not significantly affect affinity for EhUba1-EhUb.
under similar conditions (4). EhUba1, in contrast, exhibited a significantly faster initial isotope exchange velocity of 460 pmol/min (95% C.I. 428–493 pmol/min), under identical conditions. The /H110113.5-fold greater velocity of exchange for EhUba1 compared with human Uba1 is intrinsic to the E1 enzyme, given that the respective exchange rates in the presence of either human or /H11011E. histolytica ubiquitin substrates were indistinguishable (Fig. 3C). A superimposition of EhUbiquitin with the structural model of /H11011S. cerevisiae Uba1 /H11011 suggests that the divergent ubiquitin surface in /H11011E. histolytica is not utilized in the E1/ubiquitin interface (Fig. 2B), consistent with similar kinetics of the E1 enzymes in activating either the human or /H11011E. histolytica ubiquitin substrate. Residues that contact ATP in other E1 enzymes (e.g. Ref. 30), such as the GXGXXGCE motif, are well conserved in EhUba1 (not shown). A Lineweaver-Burk plot was constructed based upon PPi:ATP exchange experiments with varying ATP concentrations, allowing estimation of /H11011Km and /H11011Vmax with respect to the ATP substrate for both human and /H11011E. histolytica E1 enzymes (Fig. 3D). EhUba1 exhibited higher velocities of isotope exchange with /H11011Vmax = 459 pmol/min (95% C.I. 392–552 pmol/min), compared with its human homolog with /H11011Vmax = 115 pmol/min (95% C.I. 93–152 pmol/min) under these conditions. However, the /H11011Km values with respect to ATP were highly similar, being 45 and 50 /H9262M, respectively (Fig. 3D).

EhUba1 Engages the E2 Enzyme EhUbc5 and Transfers Activated EhUbiquitin—A number of predicted E2 ubiquitin-conjugating enzymes have been identified within the /H11011E. histolytica genome, although none has been functionally assessed (15). We cloned a subset of candidate E2s from the /H11011E. histolytica genome and attempted to express and purify each of them from /H11011E. coli. One E2 protein (Amoeba DB accession EHI_083560; Fig. 4A) with similarity to yeast Ubc4 and Ubc5 (termed EhUbc5) was highly expressed and thus selected for further study. Surface plasmon resonance was performed with immobilized EhUbc5, indicating a low affinity interaction with non-ubiquitin-associ-
ated EhUba1 (Fig. 4B). A $K_D$ value for the EhUba1/EhUbc5 interaction could not be precisely quantified by equilibrium binding analyses due to the protein concentration limitations of our assay, but was greater than 150 μM. The isolated ubiquitin-fold domain (UFD) of EhUba1 (amino acids 882–984) exhibited a similar apparent affinity for EhUbc5, indicating its sufficiency for binding the E2 (Fig. 4B). In similar SPR experiments, EhUba1 was first allowed to activate EhUbiquitin and form an EhUba1~Ub thioester complex (under conditions similar to Fig. 3A) prior to injection over an EhUbc5 surface. Following dissociation of EhUba1~Ub, persistent residual resonance was observed (Fig. 4C), suggesting that some activated ubiquitin may be transferred covalently to the EhUbc5-laden surface. Indeed, the residual resonance due to the apparent thioester-coupled ubiquitin could be rapidly eliminated by injection of the reducing agent DTT (Fig. 4C). An equilibrium binding analysis suggested at least a 10-fold greater affinity of EhUbc5 for EhUba1~Ub than for unconjugated EhUba1 (Fig. 4D). Notably, the apparent affinity of EhUbc5 for EhUba1~Ub ($K_D \approx 12$ μM) is likely underestimated by this approach, given that saturation could not be reached with each analyte injection (Fig. 4C) and the reported EhUba1~Ub concentrations (Fig. 4D) assume that 100% of the EhUba1 enzyme injected was conjugated to ubiquitin. However, correction of either potential source of error would further lower the apparent EhUba1~Ub/EhUbc5 dissociation constant value, resulting in a >10-fold preference of EhUbc5 for ubiquitin-conjugated over unconjugated EhUba1. Transfer of the EhUbiquitin thioester from EhUba1 to EhUbc5 was also demonstrated by an in vitro assay (Fig. 3A). Ubiquitin activation by EhUba1 and subsequent transfer to the E2 enzyme was found to be dependent on the presence of ATP and magnesium. The EhUbiquitin/Ub thioester bond was also sensitive to the reducing agent DTT.

**Structural Features of the E2 Ubiquitin-conjugating Enzyme EhUbc5 and Its Noncovalent Interaction with EhUbiquitin**—To gain further insight into ubiquitin conjugation in *E. histolytica*, EhUbc5 was crystallized and its structure determined by molecular replacement with diffraction data extending to 1.6-Å resolution (Table 2). Although EhUbc5 crystallized under a variety of conditions, the crystal form described here required a cobalt (II) salt. A cobalt ion could be identified in the electron density, contacting EhUbc5 molecules in adjacent asymmetric units (Fig. 5C). The cobalt ion appears to be octahedrally coordinated with two histidine and one aspartate ligand from one
Crystal Structures of EhUbiquitin and EhUbc5

EhUbiquitin interaction (Fig. 5, divergent residues predominantly reside on the protein surface hydrophobic core is identical to human UbcH5B, whereas seen in the case of EhUbiquitin, the majority of the EhUbc5 73% sequence identity and 83% similarity (Figs. 4 and 5A). As seen in the case of EhUbiquitin, the majority of the EhUbc5 hydrophobic core is identical to human UbcH5B, whereas divergent residues predominantly reside on the protein surface (Fig. 5, A and B). The β4-α2 and α2-α3 loops are highly conserved among E2 enzymes, including EhUbc5 (Fig. 4A), suggesting a likely conserved mode of interaction with covalently attached ubiquitin (Fig. 6A). In particular, EhUbc5 Cys-85 likely forms a thioester bond with the C terminus of activated EhUbiquitin (Fig. 3A). E2 enzymes are also known to bind ubiquitin-like modifiers in a noncovalent, “backside” interaction thought to be important for assembly of polyubiquitin chains (31, 32). To assess a potential noncovalent interaction between EhUbc5 and EhUbiquitin, we predicted E2 enzyme residues likely to be involved based on the known interaction between human UbcH5A and human ubiquitin (Fig. 4A) (33). The analogous residues in EhUbc5 were 62% identical and 87% similar, suggesting a potentially conserved noncovalent interaction with EhUbiquitin. In support of this hypothesis, EhUbiquitin was found to bind EhUbc5, as measured by SPR (Fig. 6, B and C). The apparent low affinity of the EhUbiquitin/ EhUbiquitin interaction ($K_D = 410 \pm 80 \mu M$) is consistent with other monoubiquitin interactions, specifically homologous noncovalent interactions with other E2 enzymes ($K_D$ values $\sim 100–500 \mu M$) (2, 31).

**EhUb5 Engages a RING Family E3 Ubiquitin Ligase**—We next sought to identify E3 ligases in *E. histolytica* that may partner with EhUb5 to ubiquitinate specific substrate proteins. The *E. histolytica* genome encodes a large number of putative E3 enzymes including HECT, RING, PHD, and U-box domain-containing proteins (15). A subset of candidate E3s were cloned from *E. histolytica* genomic DNA and expressed in *E. coli*. Of this subset, one RING family E3 and two HECT domains could be purified to near homogeneity in quantities suitable for biochemical experiments. The RING family protein (AmoebaDB accession EHI_020100), here termed EhRING1, contains predicted RING and zinc finger motifs according to SMART (34), although significant sequence divergence results in relatively high domain prediction $E$-values (Fig. 7B). We were unable to identify a clear homolog for EhRING1 in either humans or yeast. The first 246 amino acids of EhRING1 were cloned from *E. histolytica* genomic DNA and expressed in *E. coli*. Of this study were conducted with EhRING1 residues 1–246. C and D, EhUb5 bound a putative RING family E3 ubiquitin ligase from *E. histolytica* with low micromolar affinity, but not two HECT family E3 ligases, as measured by SPR. Mutation of Phe-62 drastically reduced the affinity of EhUb5 for EhRING1, indicating a likely conserved mode of E2/E3 interaction in *E. histolytica*.
maintained its ability to form a thioester bond with activated EhUbiquitin (Fig. 3A) and to bind EhUba1-EhUb (Fig. 4D), indicating proper folding of the point-mutated EhUbc5 protein. EhRING1 had only modest effects, if any, on EhUb5-catalyzed polyubiquitin chain formation, as assessed qualitatively by in vitro assays (Fig. 3B).

DISCUSSION

Our experiments demonstrate the presence of a functional ubiquitin activation and conjugation pathway in E. histolytica. The substantial differences in the EhUbiquitin protein sequence compared with other species cluster on a single surface, constructed primarily of the α1 helix. Our analyses suggest that this particular surface is not central to the structurally elucidated ubiquitin interfaces with E1s, E2s, HECT, and RING E3s, ubiquitin interacting motifs, and deubiquitinating enzymes in other species. However, the high degree of conservation at this surface in a diverse set of other organisms suggests a likely role(s) in ubiquitin functions. EhUbiquitin may have evolved to lack these functions, allowing sequence drift on the α1 helix and surrounding surface. Alternatively, EhUbiquitin may have evolved as yet undetermined alternative use for this surface. The function of the α1 helix region has not yet been established in E. histolytica; accordingly, its potential value as a therapeutic target is unclear. Of particular interest is the presence of an eighth surface lysine (Lys-54) unique to EhUbiquitin (arginine in all other organisms examined) and included in the divergent surface. Complex polyubiquitination patterns utilizing all seven surface lysines and the N terminus of ubiquitin exist in other species, corresponding to an array of interaction modes and affinities for various ubiquitin binding domains (37). Thus, it is likely that additional unique polyubiquitination patterns and interactions arise in E. histolytica, involving the additional exposed lysine. Further studies are necessary to determine the prevalence of Lys-54 polyubiquitination in E. histolytica and its potential functions.

EhUba1 appears to activate ubiquitin in a similar fashion to its homologs in other species. However, the observed significant difference in maximal velocity of PPi:ATP exchange compared with human Uba1 suggests differences in ubiquitin activation kinetics. Additional work is needed to determine whether ubiquitin activation by this enzyme is necessary for parasitic virulence, and whether specific inhibition of EhUba1 is a viable therapeutic goal. Inhibitors of mammalian E1s have been used with some success, demonstrating the feasibility of this approach (38).

EhUbc5 exhibited a striking selectivity for EhUba1-Ub compared with uncharged EhUba1. Because the EhUbc5 UFD bound EhUb5 with an affinity similar to that of uncharged EhUba1, it appears that some portion of the EhUba1-Ub complex, in addition to the UFD, contributes to E2 binding following ubiquitin activation. An altered EhUba1 conformation, or perhaps the EhUbiquitin molecule itself, may provide a higher affinity surface for EhUb5. This functionality may help EhUb5 recognize ubiquitin-bound E1 for efficient transfer and/or allow for rapid release of the E1-E2 complex once ubiquitin transfer has occurred. The noncovalent interaction of EhUbiquitin with EhUbc5 suggests a likely conserved mechanism for conjugating polyubiquitin chains (31). EhUbc5 was also seen to engage a RING family E3 (EhRING1) through a conserved mode of interaction, to the exclusion of two HECT family E3s. This finding suggests a possible RING E3 specificity for EhUbc5; however, we cannot rule out the possibility that EhUbc5 interacts with other, untested HECT E3 ligases, like its yeast homologs (39). It is unclear at this time which target proteins are ubiquitinated downstream of EhUbc5 and EhRING1.

The E. histolytica ubiquitin-proteasome pathway may provide therapeutic targets for potential treatment of amoebic colitis and amoebiasis. Of particular feasibility may be a proteasome inhibitor with selectivity for the E. histolytica protein target, given the previously demonstrated effects of proteasome inhibition on trophozoite proliferation and encystation (16). Alternatively, EhUba1-specific E1 inhibition may be expected to grossly perturb trophozoite function and viability, given the necessity of ubiquitin activation for multiple vital cellular processes in other eukaryotes (38).

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