E3 ubiquitin ligases catalyze the final step of ubiquitin conjugation and regulate numerous cellular processes. The HECT class of E3 ubiquitin (Ub) ligases directly transfers Ub from bound E2 enzyme to a myriad of substrates. The catalytic domain of HECT Ub ligases has a bilobal architecture that separates the E2 binding region and catalytic site. An important question regarding HECT domain function is the control of ligase activity and specificity. Here we present a functional analysis of the HECT domain of the E3 ligase HUWE1 based on crystal structures and show that a single N-terminal helix significantly stabilizes the HECT domain. We observe that this element modulates HECT domain activity, as measured by self-ubiquitination induced in the absence of this helix, as distinct from its effects on Ub conjugation of substrate Mcl-1. Such subtle changes to the protein may be at the heart of the vast spectrum of substrate specificities displayed by HECT domain E3 ligases.

Ubiquitin (Ub) conjugation regulates many cellular processes, including protein stability, cell cycle control, DNA repair, transcription, signal transduction, and protein trafficking (1–3). An enzymatic cascade consisting of Ub-activating enzyme (E1), Ub-conjugating enzyme (E2), and Ub ligase (E3) is responsible for catalyzing Ub conjugation to target proteins. The E1 enzyme activates Ub for transfer by adenylating its C-terminal electrophilic probe (17). After an initial biochemical characterization (7–9), HECT domains are composed of two subdomains connected by a flexible peptide linker. The N-terminal (N) lobe contains the E2 binding region, and the C-terminal (C) lobe contains the catalytic cysteine. In the structure of the E6AP-UbcH7 complex (Protein Data Bank codes 1C4Z and 1D5F), the catalytic cysteine of Ub is separated by 41 Å, suggesting that a substantial conformational rearrangement is required to achieve Ub transfer (7). Analysis of the structure of the WWP1 HECT domain (PDB code 1ND7) partially addresses how Ub is transferred from the E2 to the E3 cysteine by illustrating conformational flexibility of the HECT domain (9). In the WWP1 structure modeled in a complex with UbcH5, the C-lobe is rotated about the hinge region, placing it in closer proximity to the E2 cysteine and closing the distance between active site cysteines to 16 Å. Mutations in this hinge loop that restrict HECT domain rotation decrease activity (9). Additional structural elements within the HECT domain that modulate conformation or activity remain unknown.

HUWE1 (also called ARF-BP1, Mule, Lasu1, Ureb1, E3 histone, and HectH9) is a 482-kDa HECT domain E3 Ub ligase implicated in the regulation of cell proliferation, apoptosis, DNA damage response, and base excision repair (10–16). We recovered this enzyme in immunoprecipitations using Ub-C-terminal electrophilic probes (17). After an initial biochemical characterization (17), we completed a structural and biophysical analysis of the HECT domain to understand modulation of its robust in vitro activity. Here we present crystal structures of the HUWE1 HECT domain and characterize a structural element that both stabilizes this domain and modulates its activity. This structural element, the α1 helix, is an important component of the HECT domain that largely restricts its autoubiquitination activity while only nominally affecting Mcl-1 ubiquitination activity.
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EXPERIMENTAL PROCEDURES

Plasmids—HECT domain constructs of HUWE1 (amino acids 3993–4374 or amino acids 4012–4374) were cloned into a modified pET-28a plasmid (Novagen) containing a human rhinovirus 3C (HRV3C) protease site to generate an N-terminal His6 fusion protein for use in biochemical assays. The mutants C4341A and C4099A/C4184A/C4367A were generated using site-directed mutagenesis (Stratagene). For biochemical assays with radiolabeled substrate, FLAG-Mcl-1 (amino acids 1–327) and Ub were both cloned with an N-terminal protein kinase A site for \(^{32}P\) labeling into pET-16b and pET-28a with the HRV3C site, respectively, as previously reported (17). UBE2L3 was expressed in Rosetta (DE3) cells (Novagen). UBE2L3 was purified as a His6 fusion protein for use in biochemical assays. The mutants C4341A and C4099A/C4184A/C4367A were generated using a modified pET-28a plasmid (Novagen) containing a human His6 tag, HRV3C protease site, and with 32P-labeling into pET-16b and pET-28a with the HRV3C site, respectively, as previously reported (17). UBE2L3 was cloned into the pET-28a plasmid (Novagen).

Bacterial Protein Expression and Purification—All versions of the HUWE1 HECT domain were expressed and purified as previously reported (17). \(^{32}P\)-Labeled proteins were purified and labeled as previously described (17). Native UBE2L3 was expressed in Rosetta (DE3) cells (Novagen). UBE2L3 was precipitated from bacterial lysate by the addition of saturated ammonium sulfate to 90%. The precipitated protein was resuspended in 50 mM HEPES, pH 7.4, 200 mM NaCl, and purified by gel filtration (Superdex 75 PC 3.2/30, GE Healthcare).

Circular Dichroism—HUWE1 Δ α1 and HUWE1 + α1 HECT domains were dialyzed into 5 mM HEPES, pH 7.5, 100 mM NaCl immediately before the scanning and melting CD experiments using an AVIV model 202 CD spectrometer. HUWE1 Δ α1 HECT domain at 2.4 μM and HUWE1 + α1 HECT domain at 2.8 μM were used for scanning experiments between 195 and 280 nm at 25 °C. CD signal at 222 nm of 4.8 μM HUWE1 Δ α1 and 5.6 μM HUWE1 + α1 was recorded every 2 °C degrees over a 20–94 °C temperature ramp with 2 min of equilibration time at every step.

Biochemical Assays—Reaction mixtures (10 μl) for HUWE1 autoubiiquitination assay contained 100 nM human E1 (Ube1, Boston Biochem), 5.6 μM E2 (UBE2L3), HECT domain, and 60 μM \(^{32}P\)-labeled Ub with an ATP-regenerating system (50 mM Tris, pH 7.6, 5 mM MgCl₂, 5 mM ATP, 10 mM creatine phosphate, 3.5 units/ml creatine kinase). Reactions were incubated at room temperature, and aliquots were removed after the indicated amount of time and terminated in either 4 M urea and incubated for 15 min at 30 °C or terminated in reducing SDS-PAGE sample buffer. Samples were boiled for 10 min, separated on 10% Tris-glycine SDS-PAGE, and analyzed by immunoblot using anti-Ub antibody (Sigma).

Thioester Assay—Reaction mixtures for the thioester assay (10 μl) contained 100 nM human E1 (Ube1, Boston Biochem), 5.6 μM E2 (UBE2L3), 2 μM HUWE1 Δ4 HECT domain, and 60 μM Ub (Sigma) with an ATP regenerating system described above. Reactions were incubated at room temperature, and aliquots were removed after the indicated amount of time, terminated in 4 M urea, and incubated for 15 min at 30 °C or terminated in reducing SDS-PAGE sample buffer. Samples were boiled for 10 min, separated on 10% Tris-glycine SDS-PAGE, and analyzed by immunoblot using anti-Ub antibody (Sigma).

Single-turnover Assay—For the single-turnover assay, the E2 ~ Ub thioester was generated in a 20-μl reaction containing 200 nM E1 (Boston Biochem), 8 μM E2, the ATP regenerating system described above, 60 μM mutant Ub in which all lysines were mutated to arginine (K0 Ub) (Boston Biochem), and 1 μg/μl bovine serum albumin incubated for 25 min at room temperature. Formation of the E2 ~ Ub thioester was quenched with 50 mM EDTA on ice for 5 min. The E2 ~ Ub thioester was diluted into a chase mixture containing 2 μM HECT domain, 100 mM NaCl, 50 mM EDTA, and 1 μg/μl bovine serum albumin or the same reaction components lacking the HECT domain (labeled N in Fig. 3d). Reactions were incubated at room temperature, and aliquots were removed after the indicated amount of time and terminated in either 4 M urea and incubated for 15 min at 30 °C or in reducing SDS-PAGE sample buffer. Samples were boiled for 10 min, separated on 10% Tris-glycine SDS-PAGE, and analyzed by immunoblot using anti-Ub antibody (Sigma).

Crystallization of HUWE1 HECT Domain—Crystallization experiments with purified HUWE1 HECT domain, including the N-terminal His6 tag, HRV3C protease site, and with C4099A, C4184A, and C4341A mutations, were set up in 96-well sitting drop trays using commercially available sparse-matrix screens (Hampton Research, Qiagen). The initial crystals were improved in hanging-drop vapor diffusion setups. The HUWE1 + α1 HECT domain crystallized by mixing 1 μl of protein sample concentrated to 17 mg/ml with a 1-μl solution containing 0.1 M citric acid, pH 5.2, and 1.8 M (NH₄)₂SO₄. Birefringent crystals in the shape of thick rods with dimensions of ~80 × 40 × 40 μm grew within 2 days of incubation at 18 °C. The HUWE1 Δ α1 HECT domain crystallized by mixing a 1-μl solution containing (Na/K)₂PO₄ (pH 6.5) and 1.4 M (Na/K)₂PO₄. Thin rod-shaped crystals grew within 10 days at 23 °C.

Data Collection and Processing—For native x-ray diffraction studies, crystals were cryoprotected by soaking in 0.1 M citric acid, pH 5.2, 1.8 M (NH₄)₂SO₄, 12% glycerol for 30 s before vitrifying in liquid nitrogen. X-ray diffraction data were collected on a single cryogenized crystal at beamline 24ID-E, Advanced Photon Source (Argonne, IL), summarized in Table 1. Data were processed using DENZO and SCALEPACK (18). The crystals belong to the monoclinic space group C2 and diffracted to 1.9 Å. Initial phases were obtained by molecular replacement using PHASER from the CCP4 crystallographic program suite (19, 20), with the coordinates of the E3 ligase (Sigma). Reactions were quenched with reducing sample buffer and separated on 10% SDS-PAGE. Bands from dried gels were analyzed as above.

Mcl-1 Ubiquitination Assay—Reaction mixtures (10 μl) for the Mcl-1 ubiquitination assay were set up as above except with the addition of 5 μM \(^{32}P\)-labeled FLAG-Mcl-1 and 100 μM Ub (Sigma). Reactions were quenched with reducing sample buffer and separated on 10% SDS-PAGE. Bands from dried gels were analyzed as above.
FIGURE 1. The α helix stabilizes the HUWE1 HECT domain. a, multiple sequence alignment of helix α1 with a diverse set of human HECT E3 ligases is shown. Residue conservation is indicated by degree of shading ranging from orange (most conserved) to light yellow (least conserved). Secondary structure is illustrated with α-helices as cylinders and β-sheets as arrows. b, shown is multiple sequence alignment with a diverse set of human HECT E3 ligases indicating that sequence conservation drops off N-terminal to the α helix. The N terminus of the HECT domain α helix is indicated. c, thermostability of the HUWE1 HECT domain was measured in a CD melting experiment. HUWE1 HECT domain, α helix, WT, or with cysteine mutations, was heated in a circular dichroism cuvette, and unfolding was measured at 222 nm as a loss of helical content. Deletion of helix α helix results in a drastic reduction of thermostability. d, a CD scan experiment demonstrates the structural similarities of the +/−α helix versions of the HECT domain.
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WPP1 (PDB accession code 1ND7) as search model. The final model was refined at resolution of 1.9 Å using PHENIX with four TLS groups (9). Details of refinement are given in Table 1. The figures were made using PyMol (21).

RESULTS

Structure of the HUWE1 HECT Domain—We first attempted to crystallize the HUWE1 HECT domain using a fragment defined by the founding member of the HECT domain E3 ligase family, E6AP (PDB codes 1C42 and 1D5F) (7). The crystals diffracted to only 3.5 Å (data not shown), with fairly high temperature factors indicating vibrational disorder within the protein crystals. By sequence comparison, we noted the significance of a conserved N-terminal helix that seals the hydrophobic core of the N-lobe in the structures of WWP1, Smurf2, and Nedd4-like (residues 546–560 in WWP1 and 371–387 in Smurf2) (8, 9). The presence of this helix is conserved in over 13 HECT domain E3 ligases based on sequence comparison (Fig. 1a) and Verdecia et al. (9), highlighting its structural importance. The α1 helix has been previously described as a critical element for structural stability yet an element dispensable for HECT domain function (7, 9). Initial model-building into the 3.5-Å electron density showed a noticeable hydrophobic groove on the surface of helices 5, 11, 12, and 13, possibly indicating an additional helix being bound here. As sequence conservation drops off N-terminal of this α1 helix (Fig. 1b), we hypothesized that this element is an important part of the HECT domain. We note that expression of the HECT domain of Nedd4 yielded soluble folded product when the homologous helical segment was included in the expression construct but was not successful in its absence.7 We, therefore, asked whether the addition of helix α1 would not only assist in our crystallographic efforts but also affect the catalytic activity of the HECT domain.

The addition of helix α1 greatly stabilizes the HECT domain, as made evident in thermal denaturation experiments (Fig. 1c), shifting the transition midpoint by 16 °C from 44 to 60 °C. Although thermal stability differs between the two versions of the HECT domain, the level of secondary structure remains the same (Fig. 1d), indicating that the absence of helix α1 does not lead to unfolding but to less rigidity of the domain. We solved the structure of the helix-extended HECT domain by molecular replacement using the E3 ligase WWP1 (9) as a search model. The final model (R/Rfree 16.6/22.9%) was built and refined to 1.9 Å resolution (Table 1). The structure of HUWE1 HECT domain closely resembles that of WWP1, with which it shares 41.3% sequence identity (Fig. 2a). The HUWE1 HECT domain contains two distinct lobes similar to previously determined HECT domain structures (E6AP, Smurf2, WWP1). The larger N lobe (residues 3993–4252) contains the E2 binding region, and the smaller C lobe (residues 4259–4374) contains the conserved catalytic cysteine (C4341). The N lobe is composed of 13 α-helices and 7 β-strands, and the C lobe is composed of 4 α-helices and 4 β-strands. Residues 4253–4258 form the hinge that connects the two lobes. A rotary movement about this linker likely repositions the N and C lobes to bring the catalytic cysteine of the cognate Ub-loaded E2 in proximity to its E3 counterpart (9). Like WWP1, HUWE1 is oriented in an inverted T shape (⊥), in which the C lobe is positioned over the middle of the N lobe, with ~800 Å² of contact surface area (Fig. 2b).

Hydrogen bonds between Glu–4248 (N lobe) and Ser–4304 (C lobe) as well as Gln–4245 (N lobe) and Gln–4298 (C lobe) and a salt bridge between Glu–4246 and Lys–4295 stabilize the ⊥ conformation. The ⊥ conformation is further stabilized by water-mediated hydrogen bonds between the two lobes, involving residues Arg–4130, Gln–4147, Ser–4148, and Glu–4244 from the N lobe and Gln–4298, Thr–4340, Gly–4302, and Lys–4295 in the C lobe. The orientation of the N and C lobes of the HUWE1 HECT domain differs from the more open conformation observed in the crystal structures of E6AP and Smurf2 (Fig. 2c) (7, 8), although we cannot exclude the possibility that crystal contacts influence the observed orientation of the C lobe. The stabilizing nature of helix α1 is apparent from the extended structure, as it closes the hydrophobic core of the N-lobe (Fig. 2, a and d).

The most notable difference between HUWE1 HECT domain and previously solved structures concerns the E2 binding region (residues 4150–4200). Most of the hydro-

| TABLE 1 Data collection and refinement statistics |
|-----------------------------------------------|
| Values in parentheses are for the highest resolution shell. |
| Data set | HUWE1 HECT + α1 |
| Wavelength | 0.9793 |
| Space group | C2 |
| Cell dimensions | a, b, c (Å) |
| 119.6, 56.6, 69.6 |
| β (°) | 122.5 |
| Unique reflections | 30,847 |
| Resolution (Å) | 30.1-1.9 (1.93-1.9) |
| Rsym | 0.069 (0.392) |
| Rfree | 0.084 (0.493) |
| Completeness (%) | 98.3 (96.3) |
| Redundancy | 3.2 (2.5) |
| Wilson B factor (Å²) | 17.2 (2.1) |

1 Number of residues calculated with the program MolProbity (33).

7 E. Maspero and S. Polo, personal communication.
phobic residues in WWP1 that mediate contact with the E2 are similar to those in HUWE1, obvious from the alignment between HECT E3 ligases (Fig. 2e). The HUWE1 E2 binding region differs from that of WWP1 in that it contains additional structured elements; that is, mainly ordered β-strands not previously identified. The well ordered β-strands in the E2 binding region of the HUWE1 HECT domain extend farther from the helical core of the protein than seen in the structure of WWP1, and the loop is folded back on itself to complete the β3 strand and form the α8 helix (Fig. 2a). It is

FIGURE 2. Structure of the HUWE1 HECT domain. a, a stereo view of HUWE1 HECT domain (residues 3993–4374) shows the N and C lobes connected by the hinge loop. Helix α1 is colored green. The N-lobe contains the E2 binding region, and the C-lobe contains the catalytic cysteine (Cys-4341). b, overlay of HUWE1 (blue) and WWP1 (orange; PDB 1ND7) crystal structures is shown. c, overlay of HUWE1 (blue) and Smurf2 (orange; PDB 1ZVD) crystal structures is shown. d, helix α1 plays a significant role in mediating hydrophobic contacts that maintain the core of the HUWE1 HECT domain. Hydrophobic residues in the α1 helix, Phe-3994 and Phe-4001, pack into hydrophobic pockets in the N lobe. Arg-3998 and Asp-4009 form hydrogen bonds stabilizing the N-lobe. Lys-4014 and Tyr-4119, C-terminal to the α1 helix, orient the α1 helix to further stabilize the N-lobe. e, multiple sequence alignment of E2 binding region with a diverse set of human HECT E3 ligases is shown. Residues important for E2 binding are indicated with blue circles.
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A comparison of our two HUWE1 HECT domain structures shows that they are nearly identical with respect to the positioning of the N and C lobes (supplemental Fig. 1, a and b). For the structure lacking the α1 helix, the additional β-strands and α-helix seen in the E2 binding region remain unresolved, likely due to the low resolution data and high temperature factors.

Catalytic Activity of the HECT Domain—As the addition of the α1 helix to the HECT domain stabilized the protein, we asked whether the presence of this structural element affected HECT domain catalytic activity. We hypothesized that its addition might confer altered catalytic properties to the HECT domain compared with its helix-lacking counterpart. We, therefore, examined the ability of the HECT domain to catalyze self-ubiquitination in the presence of E1 and E2 (UBE2L3) enzymes, an ATP regenerating system, and 32P-labeled Ub (Fig. 3). The use of 32P-labeled Ub allowed us to quantify the amount of Ub adducts formed and calculate initial rates of product formation in HECT domain-limiting conditions. In this assay the HECT domain catalyzes the formation of a complex mixture of self-ubiquitinated species (Fig. 3, a and b) that are not observed in absence of the HECT domain (lane marked N). Immunoblots using an anti-His antibody confirmed that these species are ubiquitinated E3 enzyme, as it is the only species in this reaction that contains a polyhistidine tag (data not shown). The pattern of autoubiquitination observed is similar regardless of the presence of the α1 helix; both versions form multi- and polyubiquitinated species (Fig. 3, a and b). Although the pattern of product formation is similar, the presence of the α1 helix suppresses the autoubiquitination activity of the HECT domain by more than 25-fold (Fig. 3c). As autoubiquitination is observed for many Ub ligases and is often used as a criterion of E3 Ub ligase activity, we sought to further characterize the reasons for its modulation.

The autoubiquitination reaction described above produces a complex mixture of products. We examined HECT domain activity in a single-turnover reaction to monitor the first round of Ub addition to the HECT domain. This assay encompasses two steps. In the first step, E2 ~ Ub thioester is generated in a pulse reaction containing E1, UBE2L3, ATP regenerating system, and a Ub mutant in which all lysines are mutated to arginine (K0 Ub). Ub is chased from the E2 enzyme to the HECT domain added to the reaction. Ub-conjugated HECT domain is visualized by anti-Ub immunoblot. Samples were terminated in reducing or non-reducing sample buffer as indicated. The panel marked N is a chase reaction performed in the absence of HECT domain and terminated in non-reducing sample buffer.

A comparison of our two HUWE1 HECT domain structures shows that they are nearly identical with respect to the positioning of the N and C lobes (supplemental Fig. 1, a and b). For the structure lacking the α1 helix, the additional β-strands and α-helix seen in the E2 binding region remain unresolved, likely due to the low resolution data and high temperature factors.

possible that HUWE1 uses its unique E2 binding region to interact with a specific set of E2 enzymes in vivo that differ from WWP1.

FIGURE 3. E3 ubiquitin ligase activity of HUWE1 HECT domain. a and b, the autoubiquitination activity of HUWE1 HECT domain was tested using 60 μM 32P-labeled Ub as substrate and 2 μM WT Δ α1 (α) or 2.9 μM WT + α1 (b) HECT domains incubated with UBE1, UBE2L3, and an ATP regenerating system (note the different time scale for the two variants of the HECT domain). The HECT domain is omitted in the lane marked N. The asterisk denotes a likely ubiquitin polymer; the double asterisk denotes likely mono-ubiquitinated UBE2L3. Concentrations of HECT domain were chosen to obtain initial rate conditions. c, ligation activity of the WT Δ α1 and WT + α1 HECT domains in the autoubiquitination assay is shown. Activity is given as the ratio between initial velocity (pmol of total 32P-labeled Ub product/min) and total enzyme concentration E (pmol). Errors are the S.D. calculated from three independent experiments. d, shown is a single turnover assay monitoring transfer of Ub from the UBE2L3–Ub thioester to a lysine in the WT Δ α1 and WT + α1 HECT domains. The UBE2L3–Ub thioester is generated in a pulse reaction containing E1, UBE2L3, ATP regenerating system, and a Ub mutant in which all lysines are mutated to arginine (K0 Ub). Ub is chased from the E2 enzyme to the HECT domain added to the reaction. Ub-conjugated HECT domain is visualized by anti-Ub immunoblot. Samples were terminated in reducing or non-reducing sample buffer as indicated. The panel marked N is a chase reaction performed in the absence of HECT domain and terminated in non-reducing sample buffer.

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Thioester Formation in the HECT Domain—Catalysis by HECT domain E3 enzymes is a multistep process. The E3 enzyme binds Ub-loaded E2 and substrate followed by Ub transfer between the E2 and E3 catalytic cysteines. The E3 then catalyzes isopeptide bond formation between Ub and a lysine residue on the substrate, which may be the E3 itself, Ub, or another protein. We next determined whether the presence of the α1 helix affects this upstream step, in which the catalytic cysteine of the E3 enzyme forms a thioester bond with ubiquitin.

We first attempted to assay thioester formation using the wild-type HECT domain, but the enzyme efficiently catalyzes formation of the isopeptide bond on a time scale too fast to measure (23, 24). Instead, we analyzed thioester formation using a four-amino acid, C-terminal truncation of the HECT domain (referred to in the WWP1 structure as H1 1 helix) (9), which is present in the structure of the WWP1 domain-E2 interaction. After incubation of the HECT domain with E1, E2 UBE2L3, Ub, and an ATP regenerating system, the reaction was quenched with SDS-PAGE loading buffer with or without β-mercaptoethanol, and after electrophoretic resolution, was analyzed by anti-Ub immunoblot. The presence of the α1 helix greatly reduces the rate of thioester formation (Fig. 4), proportional to its suppression of autoubiquitination activity. The α1 helix, located on the back surface of the N lobe, is clearly not sufficiently close to interact with the E2 binding region of the HECT domain (Fig. 2a), suggesting that the vibrational disorder in this protein contributes to the HECT domain-E2 interaction.

Substrate Ubiquitination Catalyzed by the HECT Domain—Having seen that removal of helix α1 destabilizes the HECT domain and increases its autoubiquitination activity, we asked whether this effect is also observed during substrate ubiquitination. The anti-apoptotic Bcl-2 family member Mcl-1 is an in vivo target of HUWE1 (14). HUWE1 recruits Mcl-1 via its BH3-domain, whereas the HECT domain presented here catalyzes Mcl-1 ubiquitination. Although Mcl-1 is a substrate of the full-length HUWE1, we use this assay with the isolated HECT domain here as a measure of non-self-ubiquitination activity with an in vitro verified substrate of HUWE1. We examined initial rates of product formation under HECT domain-limiting conditions to determine whether the intrinsic activity of the HECT domain toward substrate is altered by the destabilizing effect of removing the α1 helix (Fig. 5, a and b). We find that the HECT domain-lacking α1 helix is ~5-fold more active in catalyzing Mcl-1 ubiquitination than the more stable HECT domain-containing helix α1 (Fig. 5c). These results also suggest that autoubiquitination of the HECT domain does not impair catalytic activity toward substrate. The two versions of the HUWE1 HECT domain, which differ 25-fold in autoubiquitination rates, show only a 5-fold difference in their Mcl-1 ubiquitination rates. A similar observation was made for the heterodimeric complex of the minimal catalytic domains of Ring1a/Bmi1, in which autoubiquitination of the Ring1b protein did not affect E3 ligase activity toward its substrate, histone H2A, in an in vitro reconstituted system (26). We also observe similar differences in autoubiquitination and Mcl-1 ubiquitination activity between the two versions of the HECT domain at 37 °C (Fig. 6).

Catalytic Activity of the C4341A Mutants—Mutation of the conserved catalytic cysteine to alanine (C4341A) abolishes activity of the HECT domain (supplemental Fig. 2). In the case of the helix-lacking HECT domain, we consistently observed that the C4341A mutant is capable of transferring a single ubiquitin to self (supplemental Fig. 2a) or Mcl-1 (supplemental Fig. 2c). These species were not generated when the HECT domain was omitted from the reaction (lane marked N). Although the failure of mutation of the catalytic cysteine to abolish activity has been previously observed (10), quantification of the monoubiquitinated species shows that this activity represents at best a minor fraction of wild-type activity (supplemental Fig. 2, e and f).

DISCUSSION

We present here crystal structures of the HUWE1 HECT domain and identify a conserved structural element, helix α1, that stabilizes the HECT domain and tightly modulates its activity. Helix α1 is present in the structure of the WWP1 HECT domain (referred to in the WWP1 structure as H1’) (9), where the authors note that it plays an obvious role in contributing to HECT domain stability. As the H1’ helix is oriented between the C lobe and domains N-terminal to the HECT domain that presumably mediate protein-protein interactions, the authors suggested that H1’ helix contributes to target pro-
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FIGURE 5. Substrate ubiquitination activity of the HUWE1 HECT domains. a and b, the Mcl-1 ubiquitination activity of HUWE1 HECT domain was tested using 5 μM [32P]-labeled Mcl-1 as substrate and 100 nM WT Δ α1 (a) or 300 nM WT + α1 (b) HECT domains incubated with UBE1, UBE2L3, Ub, and an ATP regenerating system. HECT domain was omitted from the lane marked N. Concentrations of HECT domain were chosen to obtain initial rate conditions. c, ligation activity of the HECT domains in the Mcl-1 ubiquitination assay is shown. Activity is given as the ratio between initial velocity (pmol of total 32P-labeled Ub product/min) and total enzyme concentration E (pmol). Errors are the S.D. calculated from three independent experiments. d and e, shown is a graph of the percent ubiquitinated Mcl-1 as a function of time in the reactions shown in panels a and b catalyzed by HUWE1 Δ α1 (d) or HUWE1 + α1 (e) HECT domains.

The α1 helix is indeed crucial for stability and identifies a role for this structural element in modulating HECT domain activity, as judged by autoubiquitination and Mcl-1 ubiquitination assays. Further experiments will determine whether this conserved helix modulates activity of other members of the HECT domain family.

In the absence of the N-terminal α1 helix, the HUWE1 HECT domain gains activity relative to its helix-extended counterpart. What could be the reason for this unexpected behavior? Deletion of helix α1 might expose hydrophilic residues that trigger assembly of HUWE1 HECT domains into oligomers. Such behavior has been observed in the crystals of E6AP, in which the C-lobe has rotated about the flexible linker that connects the two subdomains of the HECT domain (23). We favor the interpretation that removal of helix α1 destabilizes the HECT domain to produce a more relaxed version of the enzyme that exhibits greater intradomain flexibility. This increased flexibility allows the enzyme to sample more conformational states, thereby increasing its level of activity. Some of these conformational states may resemble the extended HECT domain structures observed in the crystal structures of Smurf2 and E6AP, in which the C-lobe has rotated about the flexible linker that connects the two subdomains of the HECT domain. In this scenario, removal of the α1 helix is analogous to the linker-extension mutations made in WWP1 (9). The removal of helix α1 may also shift the conformational equilibrium of the HECT domain into an orientation that facilitates the E2-HECT interaction or product release. This possibility is supported by evidence that enzymes exist in a dynamic range of conformations, and the equilibrium between these different conformers can be shifted by mutation (27).

We did not anticipate that destabilization of the HECT domain would increase enzymatic activity. The Ub transfer reaction involves defined regions including the ordered β-strands that describe the E2 binding region and the catalytic site surrounding residue Cys-4341. However, other steps, such as product release, may contribute to catalytic rate and may be influenced by increased conformational flexibility (28). A correlation between conformational flexibility and promiscuous activity has been observed for several other proteins (28). An example of a flexible enzyme is cytochrome P450, which can adopt a range of different conformations that allow it to act upon a variety of substrates. Among the P450 family of enzymes, the rigid CYP2A6 enzyme exhibits limited substrate specificity, whereas the highly flexible CYP3A4 is far more promiscuous (28). In the case of HUWE1 HECT domain, the α1 helix may serve to
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impose a constraint on the inherent flexibility of the catalytic domain, thus fine-tuning enzymatic activity.

Autoubiquitination is often used as a criterion of E3 Ub ligase activity and, for some ligases, has been proposed as a mechanism of self-regulation of stability and downstream signaling functions (29, 30). Our data show that this type of activity can be largely suppressed by minor extensions of what has been considered the core catalytic domain. We note, however, that our study focuses on the HECT domain of a multidomain protein, and there may exist other structural elements in the 482-kDa Huwe1 protein that affect its catalytic activity of Ub ligases, the functional relevance of activity. Although autoubiquitination is clear evidence of the presence of a 19-residue helix α1.

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