Substrate Filtering by the Active Site Crossover Loop in UCHL3 Revealed by Sortagging and Gain-of-function Mutations

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Determining how deubiquitinating enzymes discriminate between ubiquitin-conjugated substrates is critical to understand their function. Through application of a novel protein cleavage and tagging technique, sortagging, we show that human UCHL3 and the Plasmodium falciparum homologue, members of the ubiquitin C-terminal hydrolase family, use a unique active site crossover loop to restrict access of bulky ubiquitin adducts to the active site. Although it provides connectivity for critical active site residues in UCHL3, physical integrity of the crossover loop is dispensable for catalysis. By enlarging the active site crossover loop, we have constructed gain-of-function mutants that can accept substrates that the parent enzyme cannot, including ubiquitin chains of various linkages.

Covalent post-translational modification of proteins with the 76-amino acid ubiquitin (Ub) molecule controls many cellular processes, including protein turnover, trafficking, and transcriptional regulation (1). Ubiquitin conjugation to the ε-amino of lysine residues in target proteins is controlled by a series of enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3). Ubiquitin may also be condensed to other ubiquitin molecules, predominantly via internal lysines at positions 48 (K48) or 63 (K63) to form ubiquitin chains. The ubiquitin protein is generated as a head-to-tail fusion from the Ubb and Ubcl loci and as a linear fusion to the ribosomal protein, CEP52. Like other regulatory modifications, ubiquitination is reversible. Removal of ubiquitin is the purview of deubiquitinating enzymes (DUBs), comprised of five groups: JAMM motif proteases, ovarian tumor proteases (OTUs), ubiquitin-specific protease (USPs), Machado-Joseph disease protein domain proteases (MJDs), and ubiquitin C-terminal hydrolases (UCHs) (2, 3). Substrate recognition by these proteases is not well understood and it is highly likely that domains outside of the minimal catalytic unit regulate it. Save for members of the UCH class, no other DUBs have been crystallized in their full-length form. The UCH enzymes are proteins of modest size, capable of hydrolyzing ubiquitin adducts with small leaving groups, and contribute to homeostasis of ubiquitin levels in the cell. It is widely held that many members of this class of ubiquitin-specific hydrolases are unlikely to be involved in editing of ubiquitin-modified proteins, but rather recycle ubiquitin that has been consumed by reactions with small molecules (3). Accordingly, large N-terminal ubiquitin fusion proteins are generally poor substrates for UCH proteases in vitro (4). Because detailed structural data are available for several members of the UCH class of DUBs in their full-length form, we chose to study substrate recognition by these enzymes.

The structures of the yeast UCHL3 homologue YUH-1 (5), as well as those of the mammalian UCHL1 (6) and UCHL3 enzymes are known, for UCHL3 both in its free (7) and substrate-occupied form (8). UCHL3 is an unusual enzyme from a topological perspective: it possesses a highly knotted structure, possibly an evolutionary solution to survival in the proteolytic environment of the ubiquitin-proteasome system (9). A distinguishing feature of the enzyme’s architecture is the presence of an active site crossover loop that embraces the C-terminal segment of the ubiquitin suicide substrate with which the enzyme was co-crystalized (8). In the absence of substrate, the crossover loop is flexible and not visible in the x-ray structure. The role of this loop, and the relevance of its movements in the course of catalysis is unclear, but it has been proposed that this loop aids in the proper positioning of the substrate, a ubiquitin adduct, in the enzyme active site. In contrast to UCHL3, analysis of the UCHL1 crystal structure (51% sequence identity to UCHL3) reveals occlusion of the active site by a crossover loop that is ordered also in the absence of ubiquitin (6), but perhaps this is because of crystal packing interactions. The comparison of the UCHL1 and UCHL3 enzymes thus leaves the role of the crossover loop in catalysis or positioning of the substrate unresolved.

We engineered a cleavage site in the crossover loop of UCHL3, to explore its contribution to both structure and function. We chose to install a sortase recognition site, because it allows a site-specific cleavage and trans-acylation reaction with concomitant installation of a functionality (biotin, fluorophores) at the site of sortase cleavage (10). By applying the
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sortagging technique, we can simultaneously interrupt the connectivity of a protein peptide backbone and install a tag to track only the cleaved species. Thus, both native and cleaved sortase substrates can be tracked simultaneously in the same reaction mixture. The properties of sortagged UCHL3 inspired us to introduce yet other alterations in the crossover loop, resulting in gain-of-function mutants of UCHL3.

EXPERIMENTAL PROCEDURES

Reagents—Probe 1 was synthesized by standard N-(9-fluorenylmethoxycarbonyl) (Fmoc)-based solid phase peptide chemistry as described (10). Triglycine was purchased from Sigma. Antibodies were purchased from the following vendors: anti-hemagglutinin (HA) tag (antibody 3F10-HRP; horseradish peroxidase), Roche Applied Science; streptavidin-HRP, Amersham Biosciences; anti-FLAG tag M2 (Sigma); anti-ubiquitin (rabbit, Sigma); anti-Mcl-1 (Sigma); goat anti-rabbit-HRP (Life Sciences). Antibodies were purchased from the following vendors: anti-FLAG peptide was purchased from Sigma. Antibodies were purchased from the following vendors: anti-Mcl-1 (myeloid cell leukemia-1 protein, amino acids 1–327) (rabbit, Sigma); anti-FLAG tag M2 (Sigma); anti-ubiquitin (rabbit, Sigma); anti-Mcl-1 (Sigma); goat anti-rabbit-HRP (life Sciences). Antigen-antibody complexes were separated by SDS-PAGE or Tris/Tricine PAGE (Ub chain digestion experiments) and transferred to nitrocellulose membranes or polyvinylidene fluoride (ubiquitin chain digestion experiments). Membranes were blocked with 5% nonfat dried milk in phosphate-buffered saline supplemented with Tween 20 (PBS, 0.1% Tween 20, pH 7.4) overnight at 4°C or for 1 h at room temperature. Membranes were washed with PBST and incubated with the indicated antibodies for 1 h. Streptavidin-HRP and anti-HA (3F10-HRP) blots were then washed and developed with Western Lighting Chemiluminescence Reagent Plus (PerkinElmer Life Sciences). All other blots were washed, incubated with a goat anti-rabbit-HRP conjugate, and developed.

Sortase Cleavage and Gel Filtration of Sortagging Reactions—Sortagging of UCHL3 (10 μM) was performed by incubating with the indicated concentrations of sortase A (Srt A) in Srt buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM CaCl2), and 5 mM probe 1 in a 25-μL volume. Reactions were incubated at 37°C for 2 h and halted with sample buffer. For gel filtration, a 1-mL reaction (5 μM sortase) was purified by size exclusion chromatography on S75 Sephadex resin using 20 mM Tris, 50 mM NaCl, pH 8.0 buffer as eluent. Fractions were collected and analyzed by SDS-PAGE.

Kinetic Measurements—Ub-AMC assays were performed in assay buffer (50 mM Tris/HCl, 150 mM NaCl, 2 mM EDTA, 2 mM DTT, 1 mg/ml bovine serum albumin, pH 7.5) at 25°C. Enzyme concentrations were determined by Bradford assay (Bio-Rad). UCHL3 mediated Ub-AMC hydrolysis was performed in a total volume of 30 μL with 10 pm UCHL3 mutants and 62.5 nm Ub-AMC in a 384-well NUNC black plate. PfUCHL3 assays were performed with 25 nm enzyme and 125 nm UbAMC. Data were collected with a Spectramax M2 plate reader (Molecular Devices) with a 368 nm/467 nm filter pair and a 455-nm cutoff. Velocities were determined by fitting the initial linear data points to a least-squares regression line.

HA-UbVME Labeling and Adduct Purification—Sortase cleavage reactions (5 μM sortase) of the indicated UCHL3 mutants were performed for 2 h at 37°C and 5 μL was diluted with 7 μL of label buffer (20 mM Tris pH 8.0, 150 mM NaCl). DTT (1 mM final concentration) and 1.5 μg of HA-UbVME was added, and reactions were incubated at 37°C for 1 h. Reactions were halted with sample buffer and loaded onto 12.5% SDS-PAGE for analysis. For HA-UbVME adduct purification, a large-scale reaction (1 ml) was prepared, omitting sortase, and purified by ion-exchange chromatography as described (8).

Ubiquitin Polymer Digestion—K63-linked diubiquitin cleavage by sortagged UCHL3 Loop 2 enzyme was performed by incubating 7 μg of UCHL3 Loop 2 with either 2.25 μg or 22.5 μg of SrtA and 5 mM Probe 1 in Srt buffer for 2 h at 37°C. K63-linked diubiquitin (0.5 μg) was then added as well as 1 mM DTT and incubated for 1 h at 37°C. Reactions were halted with Tris/Tricine sample buffer and subjected to 12.5% Tris/Tricine PAGE followed by anti-ubiquitin immunoblot. Digestion of ubiquitin chains by UCHL3 loop extension mutants was performed in Srt buffer with 180 ng of each enzyme, 1 mM DTT, and 1 μg of the indicated ubiquitin chain in a total volume of 7 μL. Reactions were halted with Tris/Tricine sample buffer and loaded onto a 12.5% Tris/Tricine gel for anti-ubiquitin immu-
noblot analysis. For kinetic measurements, 500 nM of enzyme was incubated with 10 μM of either K63- or K48-linked diubiquitin in buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 2 mM DTT). At the indicated times, samples were withdrawn, quenched with Tris/Tricine loading buffer and subjected to 10% Tris/Tricine PAGE. Gels were stained with colloidal Coomassie and bands for K48 diubiquitin hydrolysis were quantitated with ImageJ. Velocities were determined by fitting the initial linear data points to a least squares regression line. Higher order K63-linked ubiquitin conjugates visible in Colloidal Blue-stained gels precluded quantitation of K63 diubiquitin hydrolysis.

**Mcl-1 Ubiquitination and Digestion**—FLAG-Mcl-1 (1 μg) was incubated with 100 ng of human UBE1 (E1), 1 μg of UbcH7

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**FIGURE 1. Characterization of UCHL3 and PFUCHL3 sortase substrates.**

**a**, crystal structure of UCHL3 in complex with UbVME (PDB: 1xd3). UbVME is in yellow with the crossover loop (blue) spanning the two halves of UCHL3 (green and orange). The position of the Loop 2 substitution is indicated by an arrow. **b**, LPETG substitution in the crossover loop renders UCHL3 and PFUCHL3 susceptible to sortase-mediated transpeptidation. Wild-type and LPETG substituted UCHL3 and PFUCHL3 mutants (10 μM) were exposed to sortase (5 μM, top blot; 150 μM, bottom blot) and biotinylated oligoglycine probe 1 (5 mM) and analyzed by streptavidin-HRP immunoblot to detect the ~20 kDa transpeptidation product. **c**, LEPTG-substituted UCHL3 and PFUCHL3 mutants catalyze Ub-AMC hydrolysis. Ub-AMC hydrolysis by wild-type and mutant UCHL3 (10 pM) was measured using subsaturating concentrations of Ub-AMC (62.5 nM). Data are plotted as the ratio of velocity to the enzyme concentration, with n = 3 (top). Enzymes with an H169A mutation are indicated by an (H), and enzymes with 5 or 10 glycines added to the crossover loop directly after the LPETG sequence are indicated by 5G and 10G. Ub-AMC hydrolysis (125 nM) was also measured for wild-type and mutant PFUCHL3 enzymes (25 pM). Data are plotted as the ratio of the velocity to the enzyme concentration, with n = 5 (bottom). **d**, an LPETG substituted UCHL3 mutant is labeled by a ubiquitin suicide substrate. HA-UbVME was titrated into a fixed amount (5 μg) of wild-type UCHL3 or the human UCHL3 Loop 2 mutant enzyme. Reactions were analyzed by 12.5% SDS-PAGE and stained with Coomassie Blue to visualize total protein.
(E2), 10 μg of Arf/BP1 (E3), and 100 μg of Ub with an ATP-regenerating system (14) for 90 min at room temperature.³ Reactions were quenched with NET buffer (50 mM Tris-HCl, pH 7.4, 0.5% Nonidet P-40, 150 mM NaCl, 5 mM EDTA) and incubated overnight at 4°C with anti-FLAG M2 antibody. Immune complexes were recovered with protein G beads (Sigma), washed extensively with NET buffer, and eluted by incubating with FLAG peptide (250 μg/ml in Srt Buffer) at 25°C with shaking for 30 min. Eluates were collected, pooled, divided, and incubated with 1 mM DTT and UCHL3 or PfUCHL3 mutant enzymes (800 ng) for 2 h at 37°C. Samples were analyzed by SDS-PAGE (10%), transferred to nitrocellulose, and anti-Mcl-1 immunoblot was performed.

RESULTS

Engineering a Sortase Cleavage Site into UCHL3—The presence of the unusual active site crossover loop in UCHL3 suggests that it may play a role in substrate selection by making sequence-specific contacts to the substrate: either to the ubiquitin (Ub) or ubiquitin-like (Ubl) moiety or to the attached leaving group. Alternatively, the crossover loop may play a role in stabilization of the catalytic center of the enzyme. The catalytic cysteine (Cys-95) and oxyanion-hole stabilizing glutamine (Gln-89) are separated from the general base histidine (His-169) and aspartic acid (Asp-184) residues by the crossover loop, with His-169 lying only three residues from the C-terminal end of the loop. The crossover loop not only traverses the active site of the enzyme, but also provides connectivity for the two halves of the catalytic center (Figs. 1a and 4a). The loop thus bridges key residues, bringing them into proximity for catalysis and possibly imparting stability to the active site. Alternatively, the active site crossover loop has been suggested to act as a substrate filter, limiting the size of the ubiquitin C-terminal leaving group (7), a possibility that has been suggested but never experimentally addressed. To examine these possibilities, we engineered an LPETG sortase cleavage site into the crossover loop of both human UCHL3 and the Plasmodium falciparum homolog, PfUCHL3. We included PfUCHL3 in our analysis because the two enzymes

³ K. R. Love, R. K. Sastry, E. Spooner, and H. L. Ploegh, manuscript in preparation.
are structurally similar, yet possess completely unrelated sequences in their crossover loop. As will be described below, results obtained for UCHL3 and PfUCHL3 are largely similar and thus allow generalization of our conclusions.

We inserted the LPETG cleavage site at three separate positions in the human UCHL3 (UCHL3) crossover loop and at two positions in the \textit{P. falciparum} UCHL3 (PfUCHL3) crossover loop (supplemental Table S1 and Fig. 1a), and expressed the mutant enzymes in \textit{Escherichia coli}. When exposed to SrtA and the biotinylated oligo-glycine nucleophile, we observed successful transacylation for all mutant enzymes, albeit at different efficiencies (Fig. 1b). The loop in both human and plasmoidium UCHL3 therefore adopts a flexible conformation in solution. The extent of cleavage of the UCHL3 Loop 2 variant by SrtA can be modulated from 10% to nearly 90% by incubation with increasing amounts of SrtA and increasing time (supplemental Fig. S1). Ubiquitin hydrolase activity was largely unchanged, as assessed by hydrolysis of the ubiquitin-7-amino-4-methylcoumarin (Ub-AMC) substrate (Fig. 1c). In addition, the LPETG substituted human UCHL3 Loop 2 mutant formed a covalent adduct with a hemagglutinin (HA)-tagged ubiquitin vinylmethyl ester suicide substrate (HA-UbVME), designed to react with the active site cysteine in DUBs, with similar efficiency as did wild-type UCHL3 (Fig. 1d). We chose to use the human UCHL3 Loop 2 and PfUCHL3 Loop 2 mutants for further analysis because these mutants retained near wild-type Ub-AMC hydrolysis activity and were most efficiently cleaved by sortase. We conducted subsequent sortagging experiments at low sortase concentrations that yield incomplete conversion to the transpeptidation product to track the properties of both cleaved and uncleaved UCHL3 species under identical conditions.

\textbf{Crossover Loop Connectivity Is Dispensable for Ubiquitin Docking—}The crossover loop in liganded UCHL3 likely assumes an ordered conformation in solution (8). When purified UCHL3-HA-UbVME adduct is exposed to SrtA, no transpeptidation is observed (Fig. 2a), consistent with previous observations that the LPETG motif must be placed in a flexible, unstructured region (10). In addition, titration of ubiquitin into the sortase cleavage reaction successfully inhibits transpeptidation (Fig. 2b). Therefore, in solution, the liganded form of UCHL3 possesses a rigid crossover loop refractory to attack by sortase. The highly knotted structure of UCHL3 suggests that it is possible to nick the crossover loop without complete unfolding of the polypeptide. We find only a slight increase in Stokes’ radius upon cleavage of the crossover loop (Fig. 3), suggesting that the nicked preparation does not undergo gross alterations in folding state. Nonetheless, the slightly larger hydrodynamic radius of the transpeptidation product is likely due to a more relaxed conformation caused by opening of the flexible crossover loop and installation of the 771-Da biotinylated probe (Fig. 3b). We further examined whether connectivity of the active site is essential for maintenance of UCHL3 structure by first cleaving the active site loop with small amounts of sortase and then delivering an HA-UbVME suicide substrate. Incomplete cleavage by sortase allows the properties of both the cleaved and uncleaved UCHL3 species to be examined simultaneously in one and the same reaction mixture. Indeed, a fraction of the cleaved UCHL3 successfully reacts with HA-UbVME, as shown by the appearance of a species that is both biotinylated and anti HA-reactive (Fig. 4b). Because the HA-UbVME adduct is refractory to cleavage by sortase, sortase-mediated transpeptidation must have preceded HA-UbVME addition. Because of the electrophilicity of the vinyl methyl ester group, we predicted that HA-UbVME adduct formation would require only the cysteine nucleophile and not the general base histidine residue. We find that mutation of His-169 to alanine results in HA-UbVME adduct formation (Fig. 4c), similar gel filtration chromatography elution profiles of uncleaved UCHL3 Loop 2 alone (10 mM) and sorted UCHL3 Loop 2 (10 mM) incubated with SrtA (5 mM) and probe 1 (5 mM), red line. Under these conditions, the majority of the UCHL3 population is uncleaved. Reactions were subjected to size exclusion chromatography on a Superdex S-75 column, and the UV absorbance at 280 nm was recorded. b: intact UCHL3 Loop 2 protein, cleaved UCHL3 Loop 2 protein, and sortase A co-migrate by size exclusion chromatography. Fractions corresponding to the major peak (60–72 ml) in the sortase cleavage reaction (red line in a) where the majority of the UCHL3 population is uncleaved were collected and analyzed by 12.5% SDS-PAGE followed by Coomassie Blue staining (top) to detect total protein or streptavidin-HRP immunoblot (bottom) to detect the transpeptidation product.

\textsuperscript{4} K. Artavanis-Tsakonas, W. A. Weihofen, R. Gaudet, and H. L. Ploegh, manuscript in preparation.
experiments (15). Moreover, DUB protein preparations typically contain a small fraction of unfolded protein that does not react with electrophilic ubiquitin derivatives (16) (Fig. 1d), despite the presence of a cysteine residue. Thus HA-UbVME reactivity reflects competency to bind ubiquitin and to position the catalytic cysteine in proximity of the electrophilic trap, and not ubiquitin hydrolase activity per se. Taken together, we conclude that connectivity of the active site crossover loop is not essential for maintenance of UCHL3 structure, as assessed by the ability of the cleaved preparation to interact with ubiquitin.

The Crossover Loop Restricts Substrate Size—We reasoned that if the role of the active site crossover loop is to restrict the size of the ubiquitin C-terminal leaving group, then ablation of the connectivity should result in relaxed leaving group specificity. Indeed, UCHL3 with a nicked active site crossover loop hydrolyzes K63-linked ubiquitin chains to monomeric ubiquitin, while the uncleaved preparation and sortase itself fail to do so (Fig. 5a). Incubation of UCHL3 with increasing concentrations of SrtA results in greater oligo-ubiquitin hydrolysis, indicating that it is the cleaved UCHL3 species that mediates liberation of mono-ubiquitin. This is consistent with the HA-UbVME labeling experiments and indicates that a portion of the cleaved UCHL3 species not only retains proper structure, but also ubiquitin hydrolase activity. The modest ubiquitin hydrolysis activity of the cleaved UCHL3 likely indicates that a fraction of the cleaved material is inactive upon transpeptidation, perhaps because the structure is subtly destabilized. Nevertheless, the gain-of-function associated with the cleaved UCHL3 preparation indicates that at least some fraction is competent to attack polyubiquitin chains.

Expansion of the Active Site Crossover Loop Results in Gain-of-function Mutants—Based on these results, we reasoned that insertion of additional amino acids in the active site crossover loop to extend it would stabilize the enzyme, while still allowing the observed relaxed substrate specificity. Accordingly, we inserted 5 and 10 glycines in the crossover loop on the background of the Loop 2 LPETG mutation. Hydrolysis of Ub-AMC was affected only mildly for both mutants (Fig. 1c). We then tested the ability of these mutants to hydrolyze ubiquitin chains of different linkages in the complete absence of sortase (Fig. 5b). Both of the UCHL3 variants with 5 or 10 added glycines in the crossover loop readily disassemble K63- and K48- linked ubiquitin chains, whereas the uncleaved UCHL3 does not. None of the mutants

![Figure 4. Crossover loop integrity is not necessary for maintenance of UCHL3 active site structure.](image-url)
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We took advantage of the known crystal structures for liganded and unliganded UCHL3 and applied the sortagging technique to a mammalian and apicomplexan representative of UCHL3. We chose to compare PfUCHL3 with its mammalian homolog because their sequences are no more than 35.9% identical, with almost no similarity in the crossover loop (20). Thus, the sequence-specific contributions of the crossover loop to catalysis can be parsed out of its structural contributions by comparison of both the host and pathogen enzymes. We have meanwhile solved the PfUCHL3 structure and found the overall fold to be virtually identical to its human counterpart. Only the unliganded form of UCHL3 is a substrate for transpeptidation; UCHL3 was not cleaved when modified with HA-UbVME prior to exposure to sortase. Combined, our results suggest that the crossover loop of UCHL3 is indeed a flexible structure, despite the ordered loop visible in the crystal structure of its relative, UCHL1. Because there is no obvious sequence similarity between the crossover loops of UCHL3 and PfUCHL3, the presence, but not the exact sequence of the crossover loop is essential to substrate selection. This conclusion is supported also by the lack of effect of the introduction of an LPETG tag at are able to hydrolyze a linear hexahistidine-tagged head-to-tail diubiquitin fusion, whereas isopeptidase-T (USP5) efficiently does so. The PfUCHL3 Loop 2 mutant was also subjected to loop expansion and incubated with the various ubiquitin polymers. The PfUCHL3 loop-expanded mutants hydrolyze K48-, and to a lesser extent K63-linked ubiquitin, but not linear ubiquitin polymers (Fig. 5c). We assessed the rates of catalysis of our engineered mutant forms of UCHL3 and PfUCHL3 and compared these to USP8 (17) and the A20 N-terminal domain (18, 19) (Fig. 6). Quantitation of K48-linked diubiquitin hydrolysis yields rates intermediate between those of USP8 and the A20 N-terminal domain (Table 1). Thus, by expanding the length of the crossover loop, we have successfully created gain-of-function versions of UCHL3 and PfUCHL3 that can hydrolyze bulky substrates that the parent molecules are unable to attack.

We tested whether the loop-expanded versions of UCHL3 can hydrolyze the proximal ubiquitin from a ubiquitinated substrate, Mcl-1 (myeloid cell leukemia-1 protein). The HECT (homologous to E6-associated protein [E6AP] C terminus) domain E3-ligase, ARF-BP1/Mule, targets the antiapoptotic Bcl-2 (B-cell leukemia/lymphoma 2 protein) family member Mcl-1 for ubiquitination at several internal lysines in vitro (14). When such ubiquitinated Mcl-1 preparations are exposed to the UCHL3 mutant with the 10 glycine expansion, the mono-ubiquitinated form of Mcl-1 is hydrolyzed (Fig. 7a). In contrast to wild-type UCHL3, substrates enjoy unfettered access to the active site of the M48<sup>USP</sup> isopeptidase. The M48<sup>USP</sup> domain, which has activity against K63- and K48-linked ubiquitin chains similar to that of the loop-expanded UCHL3 enzymes (13), also efficiently hydrolyzes the mono-ubiquitinated Mcl-1 substrate. To ensure that the observed multi-(mono)ubiquitinated forms of Mcl-1 indeed represent a single ubiquitin condensed onto several Mcl-1 lysines and not a ubiquitin chain added to a single lysine, we performed the Mcl-1 ubiquitination reaction with a ubiquitin variant incapable of polyubiquitin chain formation because it lacks lysine residues. Although the Mcl-1 ubiquitination pattern of the 13 available lysines in Mcl-1 is different with this ubiquitin variant, the UCHL3 mutant with 10 added glycines again hydrolyzes all proximal ubiquitin moieties, as assessed by a dramatic increase in the amount of Mcl-1 backbone released after digestion (Fig. 7b).

DISCUSSION

FIGURE 5. The active site crossover loop restricts ubiquitin leaving group size. a, cleavage of the crossover loop renders UCHL3 competent to disassemble ubiquitin polymers. UCHL3 Loop 2 protein (7 µg) was exposed to either 2.25 or 22.5 µg of SrtA and probe 1 (5 µl) for 2 h. Reactions were subsequently incubated with K63-linked diubiquitin chains (0.5 µg) at 37 °C for 1 h. Ubiquitin cleavage was assessed by 12.5% Tris/Tricine SDS-PAGE followed by anti-ubiquitin immunoblot. *, Ub<sub>n</sub> denotes higher order K63-linked ubiquitin in the diubiquitin preparation. b, crossover loop size expansion allows UCHL3-mediated hydrolysis of K63- and K48-linked ubiquitin but not a hexahistidine-tagged head-to-tail diubiquitin fusion. 180 ng of each enzyme (UCHL3 Loop 2; PfUCHL3 Loop 2; 5, 10 glycine insertion; 10, 10 glycine insertion; Iso-T, isopeptidase-T) was incubated with 1 µg of the indicated ubiquitin polymer for 2 h at 37 °C. Reactions were separated by 12.5% Tris/Tricine SDS-PAGE followed by anti-ubiquitin immunoblot. *, Ub<sub>n</sub> denotes higher order K63-linked ubiquitin in the diubiquitin preparation. c, loop expanded versions of PfUCHL3 hydrolyze K48-linked diubiquitin, inefficiently cleave K63-linked polyubiquitin, and are inactive against a hexahistidine-tagged head-to-tail diubiquitin fusion. 180 ng of each enzyme (UCHL3 Loop 2; PfUCHL3 Loop 2; 5, 10 glycine insertion; 10, 10 glycine insertion; Iso-T, isopeptidase-T) was incubated with 1 µg of the indicated ubiquitin polymer for 2 h at 37 °C. Reactions were analyzed as in b.

We took advantage of the known crystal structures for liganded and unliganded UCHL3 and applied the sortagging technique to a mammalian and apicomplexan representative of UCHL3. We chose to compare PIUCHL3 with its mammalian homolog because their sequences are no more than 35.9% identical, with almost no similarity in the crossover loop (20). Thus, the sequence-specific contributions of the crossover loop to catalysis can be parsed out of its structural contributions by comparison of both the host and pathogen enzymes. We have meanwhile solved the PfUCHL3 structure and found the overall fold to be virtually identical to its human counterpart. Only the unliganded form of UCHL3 is a substrate for transpeptidation; UCHL3 was not cleaved when modified with HA-UbVME prior to exposure to sortase. Combined, our results suggest that the crossover loop of UCHL3 is indeed a flexible structure, despite the ordered loop visible in the crystal structure of its relative, UCHL1. Because there is no obvious sequence similarity between the crossover loops of UCHL3 and PIUCHL3, the presence, but not the exact sequence of the crossover loop is essential to substrate selection. This conclusion is supported also by the lack of effect of the introduction of an LPETG tag at...
different positions in the crossover loop. The loop alone determines the size of the leaving group that can be liberated from ubiquitin by UCHL3. Integrity of the loop is not required for catalysis by UCHL3—the enzyme can function with its backbone cleaved very close to the active site. The stability of the enzyme is likely enhanced by the complex and knotted topology of the polypeptide backbone. There are many examples of proteins that are cleaved in unstructured loops; such cleavage is often required to generate an active enzyme from its inactive precursor, to expose the fusogenic properties of viral fusion proteins (21), or to render active the bacterial toxins of the AB type such as cholera toxin and E. coli heat labile enterotoxin (22). The cleavage of unstructured loops does not, as a rule, perturb secondary or tertiary structure for those proteins where the two forms can be examined independently. However, the processed proteins but not their precursors are often poised to undergo major structural rearrangements upon engagement of the appropriate counterstructures.

We have obtained gain-of-function mutants of UCHL3 through enlargement of the active site crossover loop. These mutants successfully disassemble both K48 and K63 ε-amine linked ubiquitin polymers, while wild-type UCHL3, which has a crossover loop diameter of ~12–15 Å, cannot. When known structures for K48- and K63-linked ubiquitin dimers are modeled in the solved UCHL3-UbVME structure (supplemental

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**TABLE 1**

| Enzyme       | V/[E] | Rate relative to USP8 | Rate relative to A20 NTD# |
|--------------|-------|-----------------------|---------------------------|
| UCHL3 + 5G   | 0.281 | 27.89                 | 195.15                    |
| UCHL3 + 10G  | 0.410 | 40.61                 | 284.19                    |
| PfUCHL3 + 5G | 0.311 | 30.80                 | 215.53                    |
| PfUCHL3 + 10G| 0.374 | 37.05                 | 259.22                    |
| USP8         | 1.009 | 100                   | 700                       |
| A20 NTD      | 0.144 | 14.27                 | 100                       |

# NTD, N-terminal domain.
Fig. S2), the proximal ubiquitin sterically clashes with UCHL3. To explain the observed hydrolysis of these substrates, the hinge region that contains the scissile Gly-Lys bond must be sufficiently flexible in solution to allow displacement of the proximal ubiquitin by UCHL3, consistent with previous observations (23). Because K63 lies near the N terminus of ubiquitin, K63-amine-linked dimers are hypothesized to have an open conformation similar to that of K63-linked diubiquitin (24). However, none of the UCHL3 or PfUCHL3 mutants can hydrolyze an α-amine-linked dimer. This does not result from a difference between the α and ε linkage itself, because UCHL3 can hydrolyze a ubiquitin fusion to the ribosomal protein CEP52, in α-linkage (4). Instead, the scissile bond in the ubiquitin head-to-tail fusion is likely inaccessible to the UCHL3 active site cysteine. When the head-to-tail ubiquitin dimer is modeled by ISG15, which has two tandem α-linked Ub domains, and fitted into the UCHL3-UbVM structure, the region where the scissile bond would be lies far outside of the active site groove (supplemental Fig. S2). Although the structural details of ISG15 likely differ from those of a ubiquitin head-to-tail fusion, we favor a model where the lack of cleavage by UCHL3 is due to differences in the conformation of K48-, K63-, and α-amine-linked ubiquitin dimers in solution.

The promiscuity of the loop-expanded UCHL3 variants provides a rationale for the properties of UCHL3. In the absence of other protein domains that target the enzyme to particular ubiquitinated proteins or ubiquitin chains of specific linkages (25), UCHL3 limits access to its active site to only those substrates that can pass through the narrow bore of the crossover loop. Thus it is unlikely that wild-type UCHL3 directly targets larger, folded proteins for deubiquitination (26).

In summary, we have reported a novel method for exploring the contribution of flexible loops to protein structure and function. By applying the sortagging technique to the active site crossover loop in UCHL3 as well as mutagenesis, we can examine the contributions of the loop to both the structure and function of the enzyme. We have expanded the range of substrates that UCHL3 can hydrolyze and provide strong support for the notion that the unusual active site crossover loop functions as a substrate filter, limiting the types of substrates that the enzyme can hydrolyze. Such modified versions of UCHL3 may be useful as general ubiquitin releasing enzymes for the study and identification of ubiquitin adducts.

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REFERENCES

1. Kerscher, O., Felberbaum, R., and Hochstrasser, M. (2006) Annu. Rev. Cell Dev. Biol. 22, 159–180
2. Love, K. R., Catic, A., Schlieker, C., and Ploegh, H. L. (2007) Nat. Chem. Biol. 3, 697–705
3. Nijman, S. M., Luna-Vargas, M. P., Velds, A., Brummelkamp, T. R., Dirac, A. M., Sixma, T. K., and Bernards, R. (2005) Cell 123, 773–786
4. Larsen, C. N., Krantz, B. A., and Wilkinson, K. D. (1998) Biochemistry 37, 3358–3368
5. Johnston, S. C., Riddle, S. M., Cohen, R. E., and Hill, C. P. (1999) EMBO J. 18, 3877–3887
6. Das, C., Hoang, Q. Q., Kreinbring, C. A., Luchansky, S. J., Meray, R. K., Ray, S. S., Lansbury, P. T., Ringe, D., and Petsko, G. A. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 4675–4680
7. Johnston, S. C., Larsen, C. N., Cook, W. J., Wilkinson, K. D., and Hill, C. P. (1997) EMBO J. 16, 3787–3796
8. Misaghi, S., Galardy, P. J., Meester, W. J., Ovaa, H., Ploegh, H. L., and Gaudet, R. (2005) J. Biol. Chem. 280, 1512–1520
9. Virnau, P., Mirny, L. A., and Kardar, M. (2006) Plos Comput. Biol. 2, e122
10. Popp, M. W., Antos, J. M., Grotenbreg, G. M., Spooner, E., and Ploegh, H. L. (2007) Nat. Chem. Biol. 3, 707–708
11. Borodovsky, A., Ovaa, H., Kolli, N., Gan-Erdene, T., Wilkinson, K. D., Ploegh, H. L., and Kessler, B. M. (2002) Chem. Biol. 9, 1149–1159
12. Ton-That, H., Liu, G., Mazmanian, S. K., Faull, K. F., and Schneewind, O. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 12424–12429
13. Schlieker, C., Weihofen, W. A., Frijs, E., Kattenhorn, L. M., Gaudet, R., and Ploegh, H. L. (2007) Mol. Cell 25, 677–687
14. Chen, D., Kon, N., Li, M., Zhang, W., Qin, J., and Gu, W. (2005) Cell 121, 1071–1083
15. Ovaa, H., Kessler, B. M., Rolen, U., Galardy, P. J., Ploegh, H. L., and Masucci, M. G. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 2253–2258
16. Hassiepen, U., Eidhoff, U., Meder, G., Bulber, J. F., Hein, A., Bodendorf, U., Lorthiois, E., and Martoglio, B. (2007) Anal. Biochem. 371, 201–207
17. Mizuno, E., Jura, T., Mukai, A., Yoshimori, T., Kitamura, N., and Komada, M. (2005) Mol. Biol. Cell 16, 5163–5174
18. Lin, S. C., Chung, J. Y., Lamothe, B., Rajashankar, K., Lu, M., Lo, Y. C., Lam, A. Y., Darnay, B. G., and Wu, H. (2008) J. Mol. Biol. 376, 526–540
19. Wertz, I. E., O’Rourke, K. M., Zhou, H., Eby, M., Aravind, L., Seshagiri, S., Wu, P., Wiesmann, C., Baker, R., Boone, D. L., Ma, A., Koonin, E. V., and Dixit, V. M. (2004) Nature 430, 694–699
20. Frickel, E. M., Quesada, V., Muething, L., Gubbels, M. J., Spooner, E., Ploegh, H., and Artavanis-Tsakonas, K. (2007) Cell Microbiol. 9, 1601–1610
21. Skehel, J. J., Cross, K., Steinhauer, D., and Wiley, D. C. (2001) Biochem. Soc. Trans. 29, 623–626
22. Chinnapen, D. J., Chinnapen, H., Saslowsky, D., and Lencer, W. I. (2007) FEMS Microbiol. Lett. 266, 129–137
23. Eddins, M. J., Varadan, R., Fushman, D., Pickart, C. M., and Wolberger, C. (2007) J. Mol. Biol. 367, 204–211
24. Pickart, C. M., and Fushman, D. (2004) Curr. Opin. Chem. Biol. 8, 610–616
25. Reyes-Turcu, F. E., Shanks, J. R., Komander, D., and Wilkinson, K. D. (2008) J. Biol. Chem. 283, 19581–19592
26. Butterworth, M. B., Edinger, R. S., Ovaa, H., Burg, D., Johnson, J. P., and Frizzell, R. A. (2007) J. Biol. Chem. 282, 37885–37893