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Specificity of the E1-E2-E3 Enzymatic Cascade for Ubiquitin C-Terminal Sequences Identified by Phage Display

Bo Zhao,[†] Karan Bhuripanyo,[†] Jeffrey Schneider,[‡] Keya Zhang,[†] Hermann Schindelin,[§] David Boone,[‡] and Jun Yin*[,†]

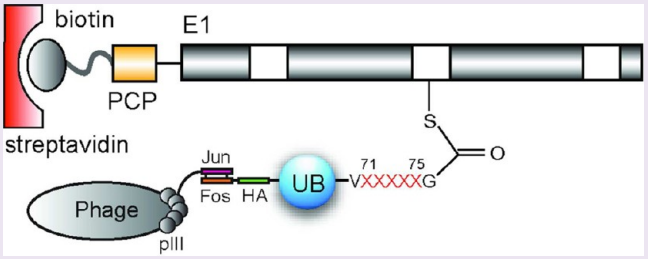
[†]Department of Chemistry, University of Chicago, 929 E. 57th Street, Chicago, Illinois 60637, United States

[‡]Department of Medicine, Committee on Molecular Pathogenesis and Molecular Medicine, Committee on Immunology, University of Chicago, Chicago, Illinois 60637, United States

[§]Rudolf Virchow Center for Experimental Biomedicine and Institute for Structural Biology, University of Würzburg, Josef-Schneider-Str. 2, 97080 Würzburg, Germany

Supporting Information

ABSTRACT: Ubiquitin (UB) is a protein modifier that regulates many essential cellular processes. To initiate protein modification by UB, the E1 enzyme activates the C-terminal carboxylate of UB to launch its transfer through the E1-E2-E3 cascade onto target proteins. In this study, we used phage display to profile the specificity of the two human E1 enzymes, Ube1 and Uba6, toward the C-terminal sequence of UB ending with ^{71}LRLRGG^{76}. Phage selection revealed that while Arg72 of UB is absolutely required for E1 recognition, UB residues at positions 71, 73, and 74 can be replaced with bulky aromatic side chains, and Gly75 of UB can be changed to Ser, Asp, and Asn for efficient E1 activation. We have thus found that the E1 enzymes have substantial promiscuity regarding the UB C-terminal sequence. The UB variants from phage selection can also be transferred from E1 to E2 enzymes; however, they are blocked from further transfer to the E3 enzymes. This suggests that the C-terminal sequence of UB is important for its discharge from E2 and subsequent transfer to E3. In addition, we observed that the Leu73Phe and Leu73Tyr single mutants of UB are resistant to cleavage by deubiquitinating enzymes (DUBs), although they can be assembled by the E1-E2-E3 cascade into poly-UB chains, thus indicating differences in UB C-terminal specificities between the E1 and DUBs. Consequently these UB mutants may provide stability to UB polymers attached to cellular proteins and facilitate the elucidation of the biological signals encoded in the UB chains.



Ubiquitin (UB) is a 76-residue protein that is attached to cellular proteins through an isopeptide bond between the C-terminal carboxylate of UB and the ε-amino group of a Lys residue on the modified protein.[1] Protein modifications by UB are associated with a myriad of cellular functions, including regulation of protein degradation, designation of protein subcellular localization, induction of protein−protein interactions, and the control of enzymatic activities. The diverse signals transduced by UB are encoded in the length and topology of the UB chains attached to the target proteins.[2,3] UB can be linked to modified proteins as a monomer or as polymers of diverse structures assembled by various isopeptide linkages between the UB molecules. To initiate protein modification by UB, an E1 enzyme first activates UB by reacting the C-terminal carboxylate of UB with ATP to form a UB-AMP adenylate.[4] Subsequently the activated UB molecule is captured by a catalytic Cys residue of E1 leading to the formation of a UB~E1 thioester ("~" is to designate the thioester bond) (Supplementary Figure 1). UB is then transferred to an E2 to form a UB~E2 conjugate *via* a thioester exchange reaction. Finally an E3 enzyme bridges the transfer of UB from E2 to the substrate proteins. Based on their mechanisms of action, the E3 enzymes can be of HECT, RING, or U-box types.[4] HECT E3s have an active site Cys residue for the formation of a UB~HECT thioester intermediate before passing the UB to the substrate proteins (Supplementary Figure 1, path a). In contrast, RING and U-box E3s function as scaffolds to recruit UB~E2 and substrate proteins to facilitate UB transfer (Supplementary Figure 1, path b). During this process, RING and U-box E3s can also be autoubiquitinated. The two E1s, about 50 E2s, and more than 1,000 E3 enzymes encoded in the human genome assemble a complex network for UB transfer pathways in the cell.[5−7] Besides UB, 17 UB-like proteins (UBL) have also been identified as protein modifiers.[8] They share a similar fold with UB and have their own E1 and E2 enzymes to facilitate their transfer to target proteins.

UB and UBLs are activated by specific E1 enzymes for protein modification.[8] The crystal structure of the UB-E1 complex revealed how the C-terminal peptide of UB plays a key

Received: July 9, 2012
Accepted: September 24, 2012
Published: September 24, 2012

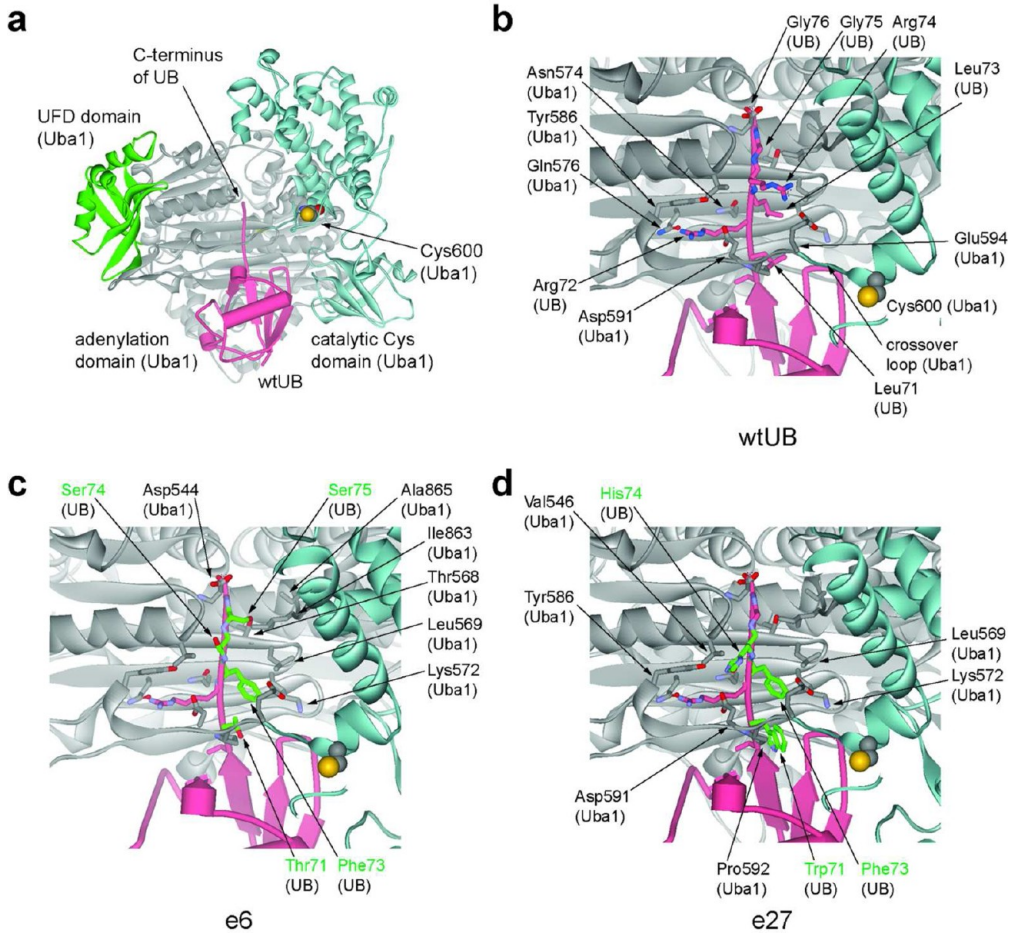


Figure 1. Structural insights into the interactions between Uba1 (E1) and phage-selected UB variants. (a) Crystal structure of Uba1 in complex with UB (PDB entry 3CMM).[9] The adenylation (A) domain of Uba1 is colored in gray, the catalytic Cys domain in cyan, and the ubiquitin fold domain (UFD) in green. UB is colored in red. (b) Enlarged view of the Uba1 active site bound to the C-terminal peptide of wtUB with the sequence ^{70}VLRLRGG^{76}. Residues of Uba1 and UB involved in critical interactions are shown. (c) Modeled structure of the UB variant e6 with the C-terminal sequence ^{70}VTRFSSG^{76} bound to Uba1. (d) Modeled structure of the UB variant e27 with the C-terminal sequence ^{70}VWRFHGG^{76} bound to Uba1. Mutated residues in e6 and e27 are colored and labeled in green.

role in UB recognition by E1 (Figure 1).[9] In this study we used phage display to profile the specificity of the E1 enzymes for the C-terminal sequence of UB. We found that E1 can activate UB variants of diverse C-terminal sequences and transfer them to E2 enzymes such as UbcH7 and UbcH5a. In contrast, the UB transfer reaction from E2 to E3 has strict requirements for the native sequence of UB. We also found that the E1-E2-E3 cascade has a different specificity for the UB C-terminal sequence compared to that of the deubiquitinating enzymes (DUBs) that catalyze UB chain disassembly.[10] On the basis of this difference, we identified UB mutants that can form poly-UB chains catalyzed by E1, E2, and E3 enzymes, yet the resulting UB chains are resistant to DUB cleavage.

RESULTS ANS DISCUSSION

Phage Display Profiling of UB C-Terminal Sequences Based on Reactivities with E1 Enzymes. The E1 enzyme activates UB and initiates its transfer through the E1-E2-E3 cascade to cellular proteins.[8] The crystal structure of the yeast E1 enzyme Uba1 in complex with UB shows that UB is bound to the adenylation (A) domain of Uba1 with its C-terminal peptide ^{71}LRLRGG^{76} extending into the ATP-binding pocket of the adenylation domain (Figure 1a).[9] This enables the C-terminal carboxylate of UB to react with ATP for the formation of a UB-AMP adenylate. A conformational change of Uba1 is then necessary to bring Cys600 of Uba1 in close proximity to the activated UB C-terminus for the formation of the UB~E1 thioester. The importance of the UB C-terminal sequence for E1 recognition was first revealed by limited proteolysis of UB in which the two C-terminal Gly residues of UB were cleaved and the truncated UB no longer supported protein degradation in cell lysates.[11,12] The roles of individual UB C-terminal residues in E1 recognition have also been studied by site-directed mutagenesis. The Gly76Ala mutant of UB was found to have very low activity with E1 because once it forms the thioester conjugate with E1, it induces a conformational change in E1 that inhibits the formation of UB-AMP at the E1 active site.[13,14] Arg72 of UB is also important for E1 binding as is manifested by the Arg72Leu mutation that increases the dissociation constant (K_d) of UB with E1 by 58-fold.[15] Mutations at the UB C-terminus also interfere with the function of UB as a protein degradation signal. Chemical modifications of Arg72 or Arg74 of UB were found to inhibit protein ubiquitination and degradation in cell lysates.[16] A systematic Ala scanning mutagenesis further revealed that mutating any of UB's C-terminal residues within ^{70}VLRLRGG^{76} to Ala has a detrimental effect on the normal growth of yeast cells.[17] Alignment of the

dx.doi.org/10.1021/cb300339p | ACS Chem. Biol. 2012, 7, 2027−2035

sequences of various UB isoforms originating from virus, yeast, plant, animal, or human sources showed that all of them have the identical C-terminal sequence of ^{70}VLRLRGG^{76}.[18] Such a high degree of sequence conservation reflects the importance of the UB C-terminus for its biological function.

Previous studies on UB interaction with E1 mainly relied on site-directed mutagenesis in which individual UB residues were mutated one at a time and the decrease in the reactivity of UB mutants with E1 was measured to probe UB-E1 recognition. To gain deeper insights into UB-E1 interaction, we wished to generate a profile of UB C-terminal sequences that are reactive toward the E1 enzyme. In this way, UB variants with alternative sequences that are nearly as active as wtUB could be identified, and the collective effects of UB C-terminal mutations on E1 catalysis could be revealed. To profile the specificity of E1 enzymes toward different UB C-terminal peptides, we designed a UB library with randomized C-terminal sequences and used phage selection to identify UB clones that are reactive with E1. Since the C-terminal Gly residue of UB (Gly76) was found to be indispensable for UB activation by E1,[13,14] we decided to leave Gly76 unchanged and randomized residues 71−75 of UB. The size of the UB library was 1×10^8, large enough to cover the sequence diversity of the UB mutants with five randomized residues (3.2×10^6). We used the human E1 enzymes Uba6 and Ube1 to carry out phage selection since it was recently reported that these two E1 enzymes have distinct specificities in transferring UB to E2s.[6]

Phage selection of the UB library was based on catalytic formation of thioester conjugates between E1 and UB variants displayed on the phage surface (Figure 2).[19] We expressed the E1 enzymes as fusions with an N-terminal peptidyl carrier protein (PCP) domain that could be labeled with biotin by Sfp phosphopantetheinyl transferase that catalyzes biotin transfer to PCP from a biotin-coenzyme A (CoA) conjugate.[20] Biotin-labeled PCP-E1 fusions were then bound to a streptavidin plate, and phage-displayed UB library was added to the plate with Mg-ATP to initiate the reaction. Phage particles displaying UB variants reactive with the E1 enzymes should be covalently bound to the plate due to the formation of a UB~E1 thioester conjugate. Catalytically active UB phage bound to the plate were then released by treatment with dithiothreitol (DTT) that cleaved the thioester linkages between the UB variants and the E1 enzyme on the plate. We carried out phage selection of the UB library with Ube1 and Uba6 in parallel. In the first round of selection, 1×10^{11} phage displaying the UB library were added to 100 pmol of the E1 enzymes immobilized in each well of the streptavidin plate, and the reaction was allowed to proceed for 1 h in the presence of 1 mM Mg-ATP for the formation of UB~E1 conjugates. In the subsequent rounds of selections, the amount of phage and the E1 enzymes, and the reaction time were decreased to make the selection more stringent (Supplementary Table 1). Eventually in the eighth round of selection, 1×10^{10} library phage were reacted with 1 pmol E1 for 10 min. During the iterative rounds of selection, we observed a substantial increase of phage enrichment from the selection reaction including both E1 and Mg-ATP over the controls in which either E1 or Mg-ATP was excluded (Supplementary Figure 2 and Supplementary Table 1). After the eighth round of selection, phage enrichment from the selection reaction was 350-fold higher than that of the controls. This indicates that catalytically active phage clones were enriched and that they should have strong reactivities with Ube1 and Uba6. UB clones from the fifth to eighth rounds of selection were sequenced, and the results are shown in Figure 3, while full alignments of UB clones selected by either Ube1 or Uba6 are summarized in Supplementary Figure 3.

Phage selections with both Ube1 and Uba6 predominantly enriched a UB clone (e27) with the C-terminal sequence ^{71}WRFHGG^{76} (Figure 3). This clone appeared 7 times out of 20 clones sequenced in the Uba6 selected phage pool and 7 times out of 60 clones sequenced among Ube1-selected phage clones. Alignment of the selected UB clones reveals a clear

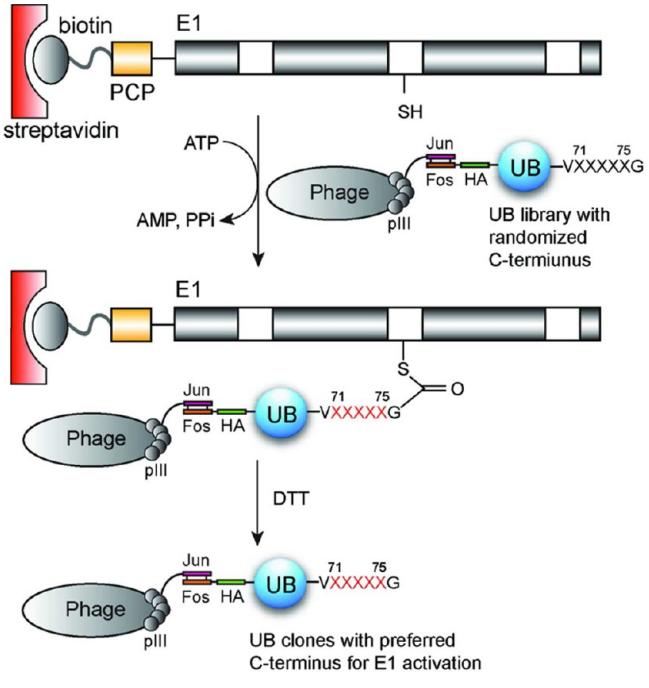


Figure 2. Phage selection of the UB library. A PCP-E1 fusion was labeled with biotin and immobilized on the streptavidin surface. Selection of the phage-displayed UB library is based on the formation of a UB~E1 conjugate. The sequence of the UB C-terminus spanning residues 71−75 was randomized. X in black denotes any amino acid residues in the UB library, and X in red denotes UB C-terminal sequences that are reactive with E1 enriched by phage selection.

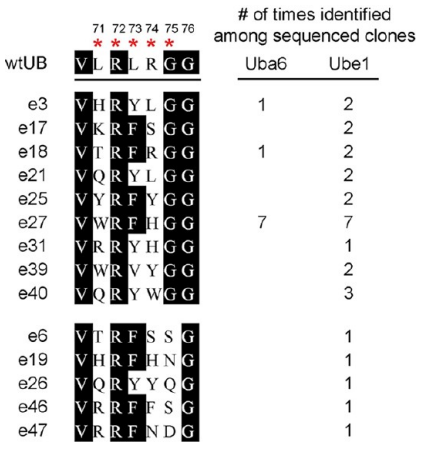


Figure 3. Alignment of the C-terminal sequences of the selected UB clones that are reactive with E1 enzymes Ube1 and Uba6. The stars designate residues that were randomized in the UB library. A full alignment of phage-selected UB clones is shown in Supplementary Figure 3.

dx.doi.org/10.1021/cb300339p | ACS Chem. Biol. 2012, 7, 2027−2035

pattern of the C-terminal sequences that are preferred substrates of E1 (Figure 3 and Supplementary Figure 3). With the exception of a single clone carrying a Val at position 72, all feature the wt Arg residue at this position, suggesting that this residue is highly critical for UB−E1 interaction. This result agrees well with mutagenesis studies showing that an Arg72Leu mutation in UB significantly impaired UB binding to E1.[15] To our surprise, residues at positions 71 and 73 of UB prefer bulky aromatic side chains such as Phe, Tyr, or Trp, quite different from the Leu residue at both positions in the wt. Positively charged residues such as Arg, Lys, and His can also be present at position 71. Residues at position 74 (Arg in wtUB) are more diverse in structure, but there is a clear preference for aromatic side chains Tyr and Phe and positively charged side chains Arg, His, and Lys. Previous mutagenesis studies showed that the Arg74Leu mutant of UB has a similar K_d with E1 as the wtUB.[15] Sequence alignments of phage-selected UB clones also show that Arg74 of UB is less critical for the UB-E1 interaction. Residues at position 75 still prefer to be a Gly to maintain the Gly-Gly motif at the very C-terminus of UB, however other residues with small polar or charged side chains such as Ser, Asp, Asn, and Gln can replace Gly75 in selected UB clones (Figure 3).

On the basis of the sequence alignments of E1-reactive UB variants, Uba6 and Ube1 have very similar selectivities for the C-terminal sequence of UB (Supplementary Figure 3). This is probably due to the high homology in the adenylation domains of both human UB-specific E1s.[6] We thus focused on Ube1 to characterize the reactivity of the phage-selected UB variants with the E1 enzyme. We initially assumed that wtUB ending with ^{71}LRLRGG^{76} should be the dominant clone in the selected phage pool, and in line with this assumption we found that 2 of the 20 clones sequenced after the fifth round of phage selection with Ube1 were wtUB. However, when we made the selection conditions more stringent in the later rounds of selection, the library clones converged to the sequences shown in the alignment (Figure 3). This indicates that the selected UB variants with alternative C-terminal sequences can compete with wtUB for E1 activation. Kinetic analysis showed that wtUB is 2−4-fold more active with Ube1 than the UB variants from phage selection (Figure 4 and Table 1), but it did not become the dominant clone in the library when the selection adopted more stringent conditions. The reason for this could be that other factors such as higher expression levels or better display might favor the enrichment of the UB variants with nonnative C-terminal sequences from the library.

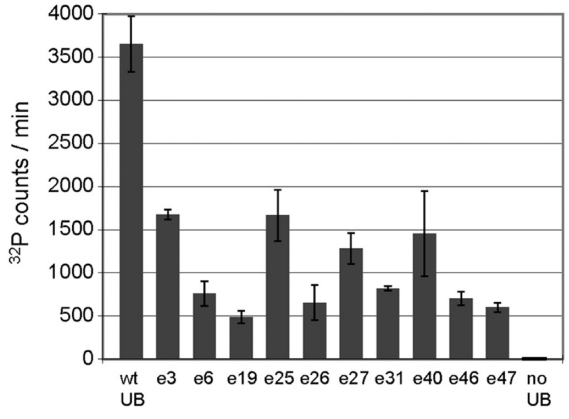


Figure 4. Activity of UB variants from phage selection with Ube1 assayed by ATP-PPi exchange.

Table 1. Kinetic Parameters of the ATP-PP$_i$ Exchange Reaction Catalyzed by Ube1 with UB Variants

	$K_{1/2}$ (μM)	k_{cat} (min^{-1})	$k_{cat}/K_{1/2}$ (μM^{-1} min^{-1})
wtUB	1.5 ± 0.5	88 ± 17	60
L73F	2.0 ± 0.7	63 ± 20	32
L73Y	1.6 ± 0.2	55 ± 11	35
e6(^{71}TRFSSG^{76})	2.5 ± 0.2	38 ± 7	16
e27(^{71}WRFHGG^{76})	4.2 ± 0.7	60 ± 15	14

Uba6 is one of the E1s used for phage selection and can also activate human leukocyte antigen F-associated transcript 10 (FAT10), a UBL protein that contains two tandem UB-like domains.[21,22] FAT10 has a C-terminal sequence of ^{160}SYCIGG^{165}, quite different from that of UB, but it has a similar activity as UB with Uba6.[23] Phage selection of the UB library did not enrich any C-terminal sequence that is homologous to that of FAT10 (Figure 3 and Supplementary Figure 3). Since the two UB-like domains of FAT10 share moderate sequence homologies of 29% and 36% with UB,[24] the native C-terminal sequence of FAT10 may need to be appended to the FAT10 scaffold to be reactive with Uba6. It would be of interest to carry out phage selection with Uba6 on a FAT10 library with randomized C-terminal sequences to see if a sequence profile close to that of UB or FAT10 emerges from the selection.

Reactivity of Phage Selected UB Variants with E1. We expressed 10 representative UB variants from phage selection and assayed their reactivities with Ube1 by ATP-PPi exchange (Figure 4).[25,26] The isotope exchange between ^{32}PPi and ATP proceeds through a UB-AMP intermediate bound to the E1 enzyme.[26−28] The apparent half-saturating concentration of UB ($K_{1/2}$) in the exchange reaction is not equal to the K_m of UB-E1 complex but is dependent on the rate of forward and reverse reactions and the equilibrium constants of various ternary complexes between E1, UB, and various ATP, AMP, and PP$_i$ species.[26] Nevertheless $K_{1/2}$ and k_{cat} of the ATP-PPi exchange reaction have been used to compare the reactivities of various UB mutants with the E1 enzyme.[11,13−16,18,29,30]

Based on the results of ATP-PPi exchange assay, we found that all UB clones showed significant activation by Ube1 with activities of ∼15−50% of that of wtUB (Figure 4). Clones ending with the Gly-Gly motif display a higher activity; for example, clones that terminate in Gly-Gly (e3, e25, e27 and e40) can be activated by Ube1 at about 50% of the activity of wtUB. These clones have an Arg72 residue but have aromatic residues Phe, Tyr, Trp or polar residues His or Gln at positions 71, 73, and 74. Clones e6, e19, e26, e46, and e47 have the penultimate Gly (Gly75) replaced by Ser, Asn, Gln, and Asp (Figure 3). They can still be activated by Ube1 but with only ∼15−20% of the activity of wtUB (Figure 4). We measured the $K_{1/2}$ and k_{cat} values of the Ube1-catalyzed activation of e27, the most abundant UB clone from phage selection (Table 1). We found that it has a similar k_{cat} value (60 min^{-1}) as wtUB (88 min^{-1}) but a $K_{1/2}$ 3-fold higher (4.2 μM) than that of wtUB (1.5 μM). For another UB clone, e6, with a Ser residue at position 75, the kinetic analysis based on ATP-PPi exchange indicates a similar $K_{1/2}$ (2.5 μM) as wtUB but a 2-fold lower k_{cat} (38 min^{-1}). This suggests that residues larger than Gly at position 75 can still be accommodated by the E1 active site to form the UB-AMP adenylate. Overall these results confirm that UB variants with quite different C-terminal sequences from

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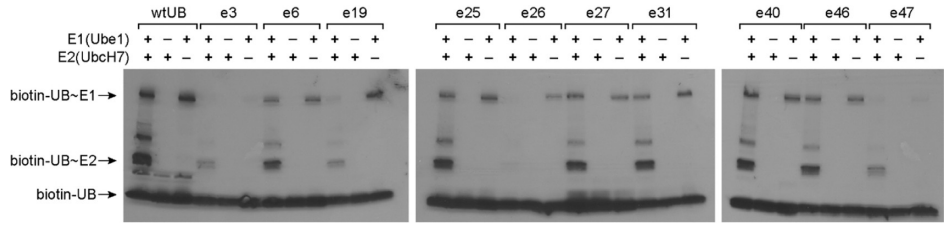

Figure 5. Western blot analysis of the formation of thioester conjugates between UB variants from phage selection and Ube1 (E1) and UbcH7 (E2). Biotin-labeled wtUB or UB variants were added in all reactions. Either Ube1 or UbcH7 was missing from the control reactions.

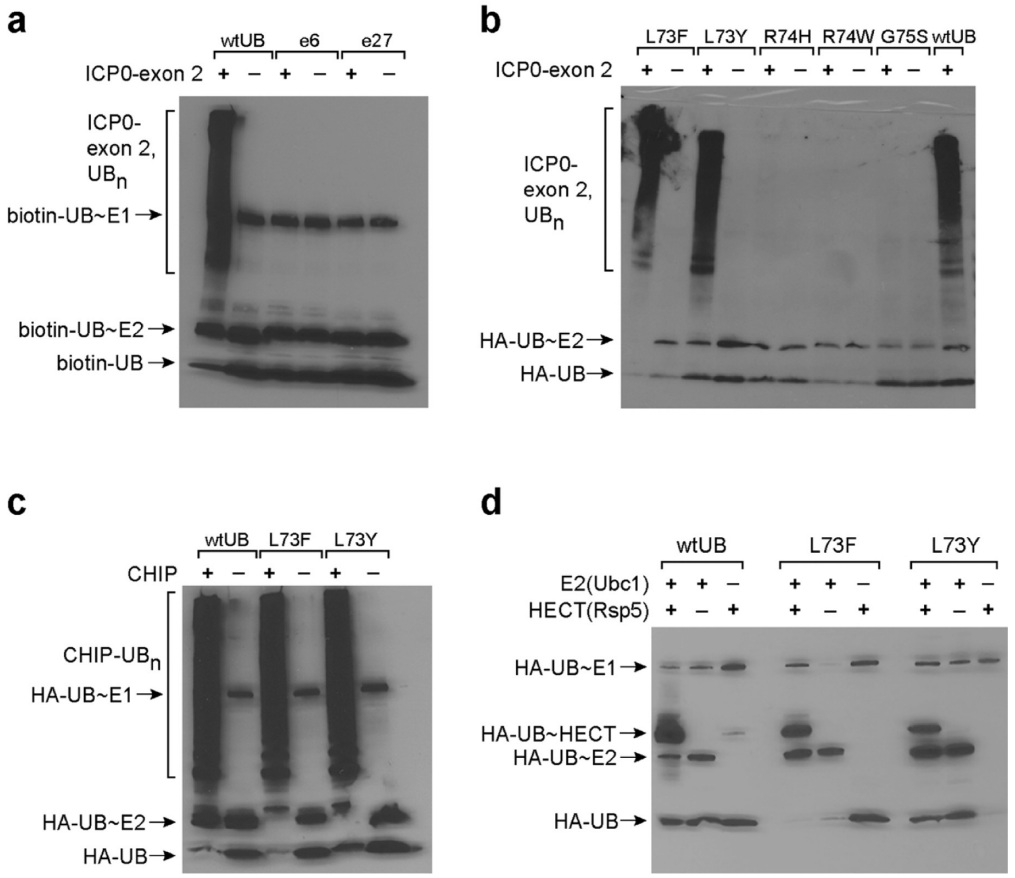

Figure 6. Reactivity of E3 with UB variants of alternative C-terminal sequences. (a) UB variants e6 and e27 from phage selection cannot be transferred to the exon 2 fragment of ICP0 E3. (b) UB variants with single mutations at the C-terminus have different reactivities in UB transfer reactions to the exon 2 fragment of ICP0. Ube1 (E1) and UbcH5a (E2) were used in the UB transfer reaction. (c) Transfer of UB mutants Leu73Phe and Leu73Tyr to CHIP E3 catalyzed by Ube1 (E1) and UbcH5a (E2). (d) Transfer of UB mutants Leu73Phe and Leu73Tyr to the HECT domain of Rsp5 catalyzed by Ube1 (E1) and Ubc1 (E2).

wtUB are catalytically active with the E1 enzymes. The high activities of the UB variants with E1 also prove the efficiency of phage display in profiling E1 selectivity with UB C-terminal sequences.

Modeling the Interactions between UB Variants and E1. Since the adenylation domains of Ube1 and Uba6 are highly homologous to that of yeast Uba1,[6] we generated models of Uba1 bound to e6 and e27 to study how E1 interacts with UB variants with alternative C-terminal sequences. The crystal structure of the UB-Uba1 complex shows that the C-terminal peptide of UB is threaded underneath the crossover loop that connects the adenylation domain and the catalytic Cys domain of Uba1 (Figure 1b). The side chains of UB residues Leu71, Leu72, and Arg74 all point toward the open space between the backbone of the UB C-terminal peptide and the catalytic Cys domain.[9] The modeled structures of the e6 or e27 complex with Uba1 show that Phe73 at the C-terminal end of e6 (^{71}TRFSSG^{76}), as well as Trp71, Phe73, and His74 at the C-terminal end of e27 (^{71}WRFHGG^{76}), fit into the active site of Uba1 without significant adjustment of the peptide backbones of either UB or Uba1 as observed in the crystal structure of the UB-Uba1 complex (Figure 1c and d). In both models, Phe73 is in close distance to the aliphatic atoms in the side chains of Leu569 and Lys572 of Uba1. The increased hydrophobic packing between Phe73 of UB variants and Uba1 residues may explain the predominant presence of aromatic Phe and Tyr residues replacing Leu73 in wtUB after phage selection.

In the crystal structure of UB-Uba1 complex, the side chain of Arg72 of UB tightly fits into a pocket composed of residues Asn574, Gln576, Tyr586, and Asp591 in the adenylation domain of Uba1 (Figure 1b). It is thus quite reasonable that phage selection overwhelmingly enriched the wt Arg residue at

dx.doi.org/10.1021/cb300339p | ACS Chem. Biol. 2012, 7, 2027−2035

position 72 in order to retain the activity of UB variants with E1 (Figure 3). The crystal structure of the UB-Uba1 complex also shows that the Cα atom of Gly75 is facing the side chains of Asp544, Thr568, Ile863, and Ala865 of Uba1 (Figure 1b,c). These residues create a narrow space that would prefer a Gly at this position although phage selection revealed that larger residues such as Ser, Asp, Asn, and Gln can replace Gly75 for E1 activation. The model of the e6-Uba1 complex suggested that a Ser residue replacing Gly75 of UB does not generate any significant steric clashes with Uba1 residues, but instead the hydroxyl group of Ser75 may be engaged in hydrogen bonding interactions with the main chain carbonyl oxygen of Thr568 of Uba1 (Figure 1c). A more detailed analysis of the modeled complexes between Uba1 and UB variants e6 and e27 is included in the Supporting Information.

Different Reactivities of the E1, E2, and E3 Enzymes toward the UB C-Terminal Sequences. We also assayed if the UB variants from phage selection can form thioester conjugates with E1 and E2s, and be further transferred to the E3 enzymes. We expressed the UB variants with an 11 residue N-terminal ybbR tag to facilitate biotin labeling of UB with Sfp and biotin-CoA.[20] We then followed the formation of UB~Ube1 (E1) and UB~UbcH7 (E2) thioester conjugates by Western blots probed with streptavidin-HRP. As shown in Figure 5, all UB variants can form thioester conjugates with Ube1 as demonstrated by the detection of a 125 kDa band, matching the combined size of UB and E1. In contrast not all UB variants from phage selection can form UB~E2 conjugates. For example, e26, with a C-terminal sequence of ^{71}QRYYQG^{76}, can form the UB~E1 conjugate but no significant UB~E2 conjugate band is generated by UB transfer from Ube1 to UbcH7. This may be due to the Gly75Gln substitution. Additionally e19 and e47 with Asn or Asp residues replacing Gly75 also have reduced formation of UB~E2 conjugates, but e6 and e46 with the Gly75Ser substitution can form UB~E2 conjugates at a normal level. Other UB variants with Gly-Gly ends (e25, e27, e31, and e40) can form UB~E2 conjugate at a similar efficiency as wtUB despite having bulky aromatic side chains flanking Arg72. These results suggest that the nonnative sequences outside of the Gly-Gly end of UB may be tolerated by E1 that catalyzes UB transfer to E2. Mutations at the C-terminal end of UB especially within the Gly-Gly motif may affect the conformational change of E1 that is crucial for its catalytic activity. In analogy to what has been observed for the SUMO E1, E1 enzymes of UB need to undergo significant conformational changes to position the catalytic Cys residue of E1 (Cys600 in Uba1) to attack the UB-AMP adenylate for the formation of UB~E1 conjugate (Figure 1a).[9,31]

We next analyzed the transfer of UB variants from E2 to (i) the catalytic domain of Rsp5,[32] (ii) the exon 2 fragment of ICP0,[33] and (iii) CHIP,[34] representing a HECT, a RING, and a U-box E3, respectively. In the presence of wtUB, the HECT domain of Rsp5 formed UB~HECT conjugates catalyzed by Ube1 and Ubc1 and likewise wtUB can also be transferred through the Ube1-UbcH5a cascade to the exon 2 of ICP0 as well as to CHIP for autoubiquitination. When we tested the UB variants in Figure 5 for transfer to the various E3 enzymes, we found that none of the UB variants can form covalent conjugates with the E3s such as Rsp5, ICP0, and CHIP. As the examples shown in Figure 6a, UB variants e6 and e27 can form thioester conjugates with the E1 and E2 enzymes; however, they cannot be transferred to ICP0 for the formation of poly-UB chains.

To probe the specificity of E2 to E3 transfer with the UB C-terminal sequence, we introduced mutations selected by phage display one at a time into UB. For example, phage selection enriched UB variants with Phe or Tyr residues substituting Leu73. We thus made Leu73Phe and Leu73Tyr point mutations of wtUB. Following this strategy we also introduced Arg74His, Arg74Trp, and Gly75Ser single mutations into wtUB. These UB mutants should be activated by Ube1 and transferred to E2s since phage-selected UB variants (e6, e27, e31, e40, *etc.*) containing these mutations can all form UB~E1 and UB~E2 conjugates (Figure 5). Indeed we see the loading of the UB single mutants on Ube1 and E2 enzymes UbcH5a and Ubc1 (Figure 6b). ATP-PPi exchange assay shows that the UB single mutants Leu73Phe and Leu73Tyr have similar activities with Ube1 as wtUB (Table 1). Regarding UB processing downstream of E2, only the Leu73 mutants of UB can be transferred to exon 2 of ICP0 (Figure 6b) and CHIP (Figure 6c) for polyubiquitination and to the HECT domain of Rsp5 for the formation of the UB~HECT conjugate (Figure 6d), whereas the UB mutants Arg74His, Arg75Trp, and Gly75Ser can neither be loaded on ICP0 or on CHIP for autoubiquitination nor transferred onto the active site cysteine of the Rsp5 HECT domain. These results demonstrate that UB transfer from E2 to E3 requires the native sequence of UB covering the C-terminal ^{74}RGG^{76}. However, the residue at position 73 that is further away from the UB C-terminus can apparently tolerate substitutions with bulky aromatic side chains. As a result, the Leu73Phe and Leu73Tyr mutant of UB can function normally in UB transfer reactions from E2 to E3s (Figure 6b–d). Overall these results reveal that the C-terminal end of UB plays a key role in UB transfer from E2 to E3.

Structural Basis of the Effects of the UB C-Terminal Mutations on E2 to E3 Transfer. C-terminal mutations of UB may affect UB interactions with E2 and E3 in a number of ways. The UB mutations may change the relative orientation of E2 and UB so that the thioester bond connecting the two is less likely to be attacked by the catalytic Cys of the HECT E3 or a Lys nucleophile on the RING or U-box E3. On the other hand, the UB mutations may also cause conformational changes of the UB~E2 conjugates and affect their binding to the E3 enzymes. So far quite a few structures of UB-E2 complexes have been solved by X-ray crystallography or NMR, and they reveal that UB can be bound to E2 through multiple interfaces that lead to "open", "closed" and "backbent" orientations of the UB~E2 complex.[35−42] Nevertheless, mutations in UB or E2 at their binding interface have been found to stabilize the UB~E2 conjugate and make it less reactive with the Lys residues on an E3 or on an acceptor UB. For example, mutations at the UB binding sites of Ube2S, Cdc34 and UbcH5c affect the discharge of UB from the UB~E2 conjugates and inhibit the formation of poly-UB chains.[43−45] Mutations of Leu8, Ile44 and Val 70 in UB that form the hydrophobic patch also affect the binding of UB to E2 and decrease the reactivity of the UB~E2 thioester.[44,45] We thus propose that the UB C-terminal residues may change the structures of the UB~E2 conjugates and hence decrease its reactivity. Indeed the UB C-terminal residues have been shown to be involved in multiple interactions with E2. In the crystal structure of a UB~UbcH5b conjugate in complex with the HECT domain of Nedd4L, Arg74 of UB forms a hydrogen bond with the hydroxyl group of Ser91 of UbcH5b (Supplementary Figure 4a).[39] The modeled structure of the Ubc1~UB conjugate also revealed extensive contacts between

Ubc1 and C-terminal residues of UB spanning ^{75}LRLRGG^{76} (Supplementary Figure 4b).[38]

Mutations in the UB C-terminus may also change the binding of UB to the E3 enzyme. In the complex structure of UbcH5c~UB with the Nedd4L HECT domain, UB residues Leu71 and Leu73 make contacts with the C-terminal portion of the HECT domain (Supplementary Figure 4a).[39] In a modeled structure of the RING domain dimer with UbcH5b~UB conjugate, the RING domain of the E3 enzyme RNF4 binds to the hydrophobic patch (Leu8, Ile44, and Val 70) of UB.[46] Conceivably, the UB C-terminal mutations may distort the UB orientation relative to E3 and block UB transfer from E2 to E3. The strict requirement of the E2 to E3 transfer reaction with the UB C-terminus may also be the reason why UB isoforms from different species all have the identical C-terminal sequence.[18] More structural information on UB interactions with E2 and E3 enzymes during the transfer reaction should help to elucidate the critical roles of UB C-terminus on regulating the activity of UB transfer through the E2-E3 cascade.

Reactivity of DUB with UB C-Terminal Mutants. Previous studies found that human DUB enzymes such as IsoT (USP5), UCH-L3, OTU-1, and viral PLpro all have strict specificity for the UB C-terminus ending with ^{73}LRGG^{76}.[47] This was manifested by screening the activities of DUBs to cleave an amide linkage between a fluorophore and the C-terminus of a tetramer peptide library with sequences differing from ^{73}LRGG^{76}. It has been shown that with Gly76 being fixed, any variation of the ^{73}LRG^{75} sequence leads to significantly decreased cleavage efficiency by the DUB enzymes.[47] In another study the Leu73Ala mutation on UB resulted in a 100-fold decrease in the rate of cleaving a Trp residue from the C-terminus of UB by the DUB enzyme UCH-L1.[48] Although site-directed mutagenesis has identified UB mutants that are resistant to DUB cleavage, it is not obvious from the reported studies that the corresponding UB mutants retain normal activities with the E1-E2-E3 cascade for the assembly of poly-UB chains. Since we found that the Leu73Phe and Leu73Tyr mutants of UB can function normally for UB chain formation, we tested if the UB chains with Leu73 mutations were resistant to DUB cleavage.

The exon 2 fragment of ICP0 has previously been reported to catalyze the formation of free poly-UB chains in the presence of E1 enzyme and UbcH5a as the E2 enzyme.[49] We thus assembled polymers of wtUB or UB mutants by ICP0 exon 2 in the presence of Ube1 and UbcH5a. It was found that the UbcH5 family of E2s can generate poly-UB chains of mixed linkage types.[50] We thus expected that the UB chains generated by UbcH5a and ICP0 exon 2 were connected by isopeptide bonds involving various Lys residues of UB. When poly-UB chains formed by ICP0 were treated with a viral DUB, the papain-like protease (PLpro) from the severe acute respiratory syndrome-corona virus (SARS-CoV),[51] we observed complete disassembly of wtUB chains by PLpro within half an hour, while UB chains of the Leu73Phe and Leu73Tyr mutants were resistant to PLpro cleavage after 2 h of reaction (Figure 7). Similarly, wtUB chains were significantly digested after 16 h by IsoT, a USP family of DUB,[52] while chains of the mutant UBs were still stable with the same exposure to IsoT. These results suggest that the Leu73Phe or Leu73Tyr mutants of UB are resistant to DUB cleavage, although they can be transferred normally to E3 for polyubiquitination. The inhibitory effect of the Leu73Phe and Leu73Tyr mutations of UB on DUB cleavage matches with the crystal structures of the DUB enzymes that show well-defined binding sites for the Leu73 residue of wtUB. A detailed analysis of DUB structures for Leu73 recognition can be found in the Supporting Information.

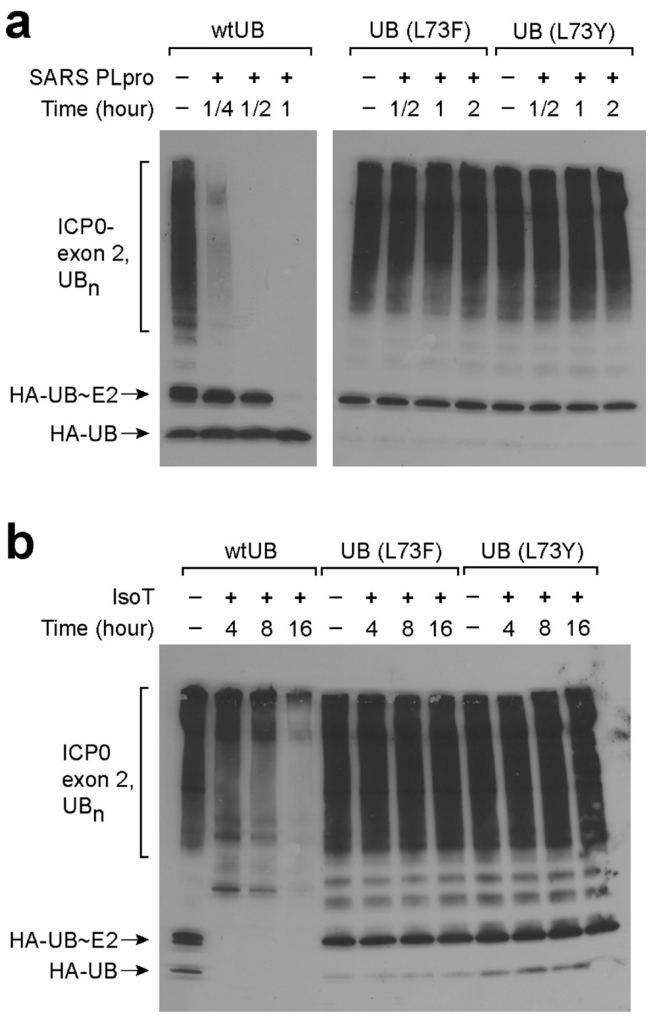

Figure 7. UB polymers formed by the Leu73Phe or Leu73Tyr mutants of UB are resistant to DUB cleavage. DUB enzymes SARS PLpro (a) and IsoT (b) were used to cleave the polymers of wtUB and mutant UB formed by Ube1, UbcH5a, and the exon 2 fragment of ICP0. The cleavage reactions were allowed to proceed for variable lengths of time, and the extent of UB chain cleavage was analyzed by Western blot probed with an anti-HA antibody.

Conclusions. In this study we developed a phage selection method to profile the specificity of the human E1 enzymes Ube1 and Uba6 with the UB C-terminus with the native sequence ^{70}VLRLRGG^{76}. Our results showed that the E1 enzymes can activate UB variants with surprisingly diverse C-terminal sequences and transfer them to the E2 enzyme. In contrast UB transfer from E2 to E3 has a strict requirement for the native C-terminus of UB with the sequence ^{74}RGG^{76}. We further found that the Leu73Phe and Leu73Tyr single mutants of UB can form poly-UB chains by the E1-E2-E3 cascade but that the resulting UB chains are resistant to DUB cleavage. Consequently these UB mutants may stabilize UB chains of various topologies in the cell in the presence of the cellular DUBs. We carried out phage selection of the UB library based on the catalytic formation of UB~E1 conjugates. Since various UBLs react with their cognate E1s to form UBL~E1

dx.doi.org/10.1021/cb300339p | ACS Chem. Biol. 2012, 7, 2027−2035

conjugates, the same phage selection strategy can also be used to profile the substrate specificities of the E1 enzymes of UBLs such as Nedd8, SUMO and ISG15.

METHODS

Phage Selection of the UB Library. In the first round of phage selection, UB transfer to immobilized Uba6 or Ube1 was set up in a streptavidin plate coated with 100 pmol PCP-E1 fusion attached to biotin. A 100 μL portion of phage solution containing about 1×10^{11} phage in reaction buffer (TBS with 1.5% BSA, 1 mM ATP, and 50 mM $MgCl_2$) was added to the plate, and the reaction was allowed to proceed for 1 h at RT. Next, the supernatant was discarded, and the plate was washed 30 times with 200 μL of 0.05% (v/v) Tween 20, 0.05% (v/v) Triton X-100 in TBS (TBS-T) and 30 times with TBS per well. After washing, phages bound to the streptavidin surface were eluted by adding 100 μL of 20 mM dithiothreitol (DTT) in TBS to each well and incubating for 10 min. The eluted phage were combined, added to 10 mL of log phase *E. coli* XL1-Blue cells, and shaken at 37 °C for 1 h to infect the cells. The cells were then plated on LB agar plates supplemented with 2% (w/v) glucose and 100 μg/mL ampicillin. After overnight incubation at 37 °C, colonies on the plates were harvested, and the phagemid DNA was extracted with a QIAprep Plasmid Miniprep kit (Qiagen). The phagemid DNA was then used for the next round of phage amplification and selection. After each round of selection, phage particles eluted from the selection or the control reactions were titered. During iterative rounds of selection, the number of the input phage particles, the concentration of E1 enzymes, and the reaction time were decreased in each round to increase the selection stringency. Eventually, 10^{10} phage particles in 100 μL reaction were incubated with 1 pmol E1 immobilized on a streptavidin coated well for 10 min at RT for the eighth round of selection. After the fifth round of selection, phage clones were sequenced with the Jun13 primer.

ASSOCIATED CONTENT

Supporting Information

Structural analysis of modeled complexes between Uba1 and UB variants e6 and e27, discussion of DUB recognition of Leu73 of wtUB, supplementary figures, and full methods. This material is available free of charge *via* the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: junyin@uchicago.edu.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by a lab startup grant from the University of Chicago, and a National Science Foundation CAREER award (1057092) to J.Y. and the Deutsche Forschungsgemeinschaft (FZ82 and Schi425/5-1) to H.S. This work was also funded in part by the Chicago Biomedical Consortium with support from the Searle Funds at The Chicago Community Trust to J.Y. We thank C. Barbas (Scripps), W. Harper (Harvard), B. Roizman (U Chicago), S. Conzen (U Chicago), J. Huibregtse (U Texas Austin), L. Hicke (Northwestern U), C. Patterson (U North Carolina), and K. Wilkinson (Emory U) for generously providing materials for this research. We also thank K. Hofmann (U Cologne, Germany), B. Schulman (St. Jude Children's Research Hospital), and N. Zheng (U Washington) for helpful discussions.

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