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Linkage-Specific Synthesis of Di-ubiquitin Probes Enabled by the Incorporation of Unnatural Amino Acid ThzK

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Di-ubiquitin (diUB) conjugates of defined linkages are useful tools for probing the functions of UB ligases, UB-binding proteins and deubiquitinating enzymes (DUBs) in coding, decoding and editing the signals carried by the UB chains. Here we developed an efficient method for linkage-specific synthesis of diUB probes based on the incorporation of the unnatural amino acid (UAA) N^{ε}-L-thiaprolyl-L-Lys (L-ThzK) into UB for ligation with another UB at a defined Lys position. The diUB formed by the UAA-mediated ligation reaction has a G76C mutation on the side of donor UB for conjugation with E2 and E3 enzymes or undergoing dethiolation to generate a covalent trap for DUBs. The development of UAA mutagenesis for diUB synthesis provides an easy route for preparing linkage-specific UB-based probes to decipher the biological signals mediated by protein ubiquitination.

Ubiquitin (UB) chains are conjugates of the 76-residue UB protein through (iso)peptide linkages between the C-terminal carboxylate of one UB and one of the seven Lys residues or the N-terminal amino group of another UB.[1] UB chains of different linkages present distinctive interfaces for recognition by the partner proteins, which make them carriers of diverse signals that are stored in the linkage type and length of the UB chains, and in the cellular targets with which the UB chains are anchored.[2] It has been a challenge to synthesize UB chains of defined linkages to identify their partner proteins such as proteins with UB-binding domains (UBDs) and deubiquitinating enzymes (DUBs) as readers and editors of the chain-encoded signals.[1,3] diUB conjugates of just two isopeptide-linked UBs are the simplest and easiest accessible prototypes of UB chains, so they have been the focus of many studies to generate linkage-defined diUB probes to decipher cell signaling mediated by protein ubiquitination.[4] Over the years, many methods have been developed for the synthesis of diUBs, including enzyme-mediated UB conjugation and total synthesis of UB conjugates of all linkage types by solid-phase peptide chemistry.[5] Recently, a handful methods have been developed taking advantage of an expanded protein coupling capacity enabled by the incorporation of unnatural amino acids (UAA) into proteins.[6] For example, propargyl Lys (PlK) and p-azido phenylalanine (AzF) could each be used to replace specific Lys residues in UB for conjugation with another UB molecule by click chemistry.[7] Another method known as "genetically encoded orthogonal protection and activated ligation" (GOPAL) was developed for incorporation of a Lys analogue with a Boc protected ε-amino group (BocK) into UB facilitated by the pyrrolysine tRNA synthetase (Pyl-RS).[8] After BocK replacement of a specific Lys residue of UB, the rest of the amino functionalities in UB, including the N-terminal amine and the Lys residues, were globally protected with Cbz or Alloc groups that are orthogonal to BocK for deprotection. Selective Boc deprotection exposes a specific Lys for coupling with the carboxylate end of another UB to furnish the linkage-specific synthesis of a diUB conjugate. Pyl-RS has also been used to accommodate Cys-conjugated Lys (CysK) for its incorporation into proteins replacing a specific Lys. CysK incorporation adds a 1,2-aminothiol functionality for coupling with the UB C-terminal carboxylate by expressed protein ligation.[9] Furthermore, Pyl-RS has been engineered for incorporating ThzK (Scheme 1, **1**), a thiazolidine conjugated Lys derivative, into proteins, and once incorporated, ThzK can be converted to CysK to provide the functionality for protein ligation.[10] We previously reported the use of methyl ester form of ThzK (L-ThzK-OMe) in *E. coli* cell culture for enhancing the yield of ThzK-incorporated UB.[11] Here, we report that ThzK replacing individual Lys residues (K11, K48, and K63) in UB can be converted to CysK and used

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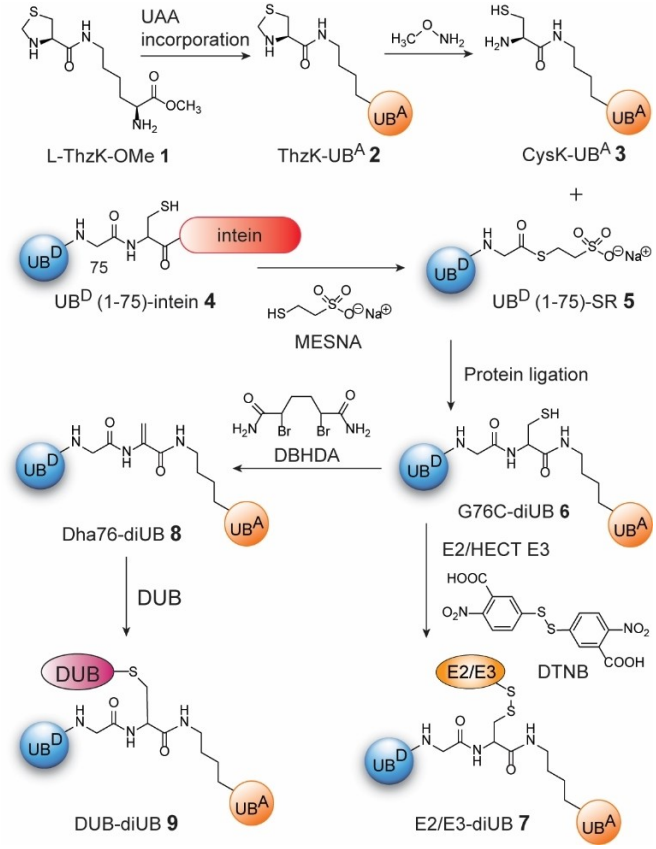

Scheme 1. Synthesis of linkage-specific diUB probes that are reactive with E2/E3 enzymes and DUBs.

for the synthesis of diUB of defined linkages by protein ligation. We further demonstrate that the Cys residue left by the ligation reaction at the diUB conjunction site can form disulfide conjugates with the catalytic Cys residues of E2 or HECT E3s or undergo dethiolation to generate a reactive dehydroalanine (Dha) functionality to trap DUBs.

We designed a scheme for the linkage-specific synthesis of diUB probes taking advantage of ThzK incorporation (Scheme 1). We first express UB with a Lys-to-ThzK mutation at a defined position (**2**). Treatment of ThzK-incorporated UB (**2**) with O-methylhydroxyamine converts ThzK to a CysK residue (**3**), enabling its ligation with UB thioester **5** to generate the diUB conjugate **6**. To prepare **5**, we expressed UB (1–75) missing the C-terminal G76 residue as an intein fusion tagged with a chitin-binding domain (**4**) and purified the fusion protein by chitin affinity chromatography. On-column cleavage of the intein with sodium 2-mercaptoethanesulfonate (MESNa) releases the UB~SR conjugate **5** from the chitin resin. We then reacted **5** with CysK-UB **3** to generate diUB with a defined isopeptide linkage (**6**) between the donor (UB^D) and acceptor UB (UB^A) by expressed protein ligation. The ligation reaction leaves behind a G76C mutation on the donor UB side of the isopeptide linkage. The newly installed Cys sidechain can form covalent conjugates with E2 or E3 enzymes (**7**) or undergo thiol elimination facilitated by 2,5-dibromohexane diacetamide (DBHDA) to generate Dha76-diUB **8** that can form covalent conjugates with DUBs (**9**).

For expressing the UB K48ThzK mutant by UAA incorporation, we transformed *E. coli* cells with the plasmids pBK-ThzKRS, encoding the engineered Pyl-RS of the *Methanosarcina barkeri* (*Mb*) origin,[10] and pMyo-UBK48TAG-pylT, encoding the Pyl tRNA and UB with N-terminal 6×His and HA tags and with the K48 codon replaced by the Amber codon. We cultured the cells in media supplemented with 1 mM L-ThzK-OMe **1**. As previously reported,[11] we could express the UB K48ThzK mutant with a yield of more than 20 mg per liter of cell culture, determined as the amount of protein eluted from the Ni-NTA column (Figure 1A).

UB K48ThzK **2** was then treated with O-methylhydroxyamine to generate the UB K48CysK mutant **3**, and the conversion of the thiazolidine ring to the 1,2-aminothiol functionality was confirmed by the decrease of the MW of UB as measured by MALDI-MS (Figure S1A–B). To prepare the UB~SR conjugate **5**, we expressed the UB-intein fusion in *E. coli*, affinity-purified the fusion protein from the cell lysate by chitin affinity chromatography, and performed on-bead cleavage of the fusion protein with MESNa (Figure 1B and Figure S1C), as described above. The reaction between UB K48CysK and UB~SR was found to be efficient. Instant formation of the diUB conjugate **6** was observed once **3** and **5** were mixed together, and incubation at room temperature for 2 hours drove the reaction to completion (Figure 1C). We subsequently purified the diUB conjugate by Ni-NTA affinity chromatography to remove excess UB~SR (Figure 1D). Following the same protocol, we expressed UB K11ThzK and UB K63ThzK mutants with a

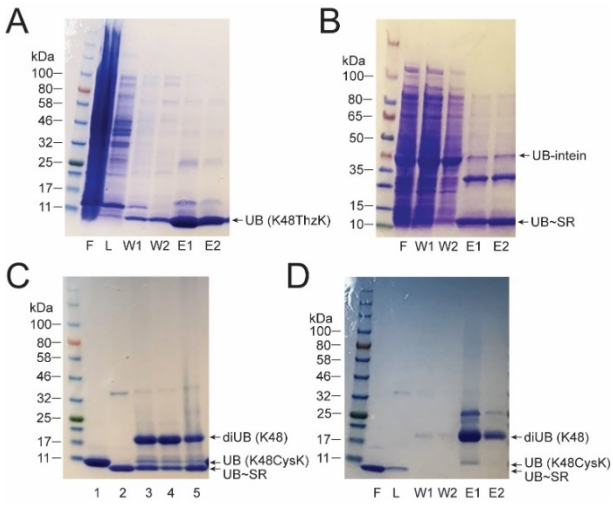

Figure 1. Synthesis of diUB conjugate with the K48 linkage. (A) Expression and purification of UB with the K48ThzK mutation by Ni-NTA affinity column (F, flowthrough, L, lysis buffer wash, W1 and W2, wash buffer wash, E1 and E2, elution). (B) Expression of UB(1-75)-intein fusion and cleavage of the fusion protein on chitin beads to generate UB~SR (F, flowthrough, W1 and W2, wash, E1 and E2, elution by MESNa). (C) Synthesis of K48 diUB by protein ligation. 1, UB with the K48CysK mutation; 2, UB~SR, 3, ligation reaction after instant mixing; 4, ligation for 2 hours; 5, ligation for 4 hours. (D) Purification of K48 diUB by the Ni-NTA affinity column. Same lane assignment as in (A).

high yield (~20 mg/L cell culture), converted the ThzK residue to CysK, and performed ligation with UB~SR to generate diUB conjugates of K11 and K63 linkages (Figure S2). The typical yield of diUB conjugate from the ligation of 10 mg of the UB CysK mutant **3** and 10 mg UB~SR **5** was about 8 mg after Ni-NTA purification. In order to verify the diUB linkage, we analyzed a sample of K11-linked diUB by MS/MS after tryptic digestion and detected a peak matching the expected peptide mass in the +3 charge state. When this peak was captured and fragmented, we identified fragment ions consistent with linkage formation at the K11 site, as expected (Figure S3).

The G76C mutation at the hinge of diUB provides a thiol-reactive handle for covalent attachment to the catalytic Cys residues of E2s and HECT E3s (Scheme 1).[12] Such ternary complexes stabilize the interaction of the donor and acceptor UB with the enzymes and may be useful for elucidating the enzymatic mechanism of UB chain synthesis. We first focused on the catalytic domain of the K11-linkage specific human E2 Ube2S as a model system. We treated a purified single-cysteine variant of this domain (C118A) with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) to form a mixed disulfide conjugate with the catalytic Cys (C95) residue following a reported procedure.[13] Subsequently, K11-diUB with the G76C mutation was added, and an E2-diUB conjugate was formed through disulfide exchange. Indeed, we observed the conjugation/coupling of Ube2S with K11-diUB at a varying ratio of the two components (Figure 2A). We also applied our strategy successfully to the conjugation of K48-diUB to the HECT domain of Huwe1 and K63-diUB to the HECT domain of Nedd4 and observed the formation of the diUB conjugates with the HECT domains (Figure 2B–C). Note that both HECT domains were supplied as single-cysteine variants with the catalytic Cys retained for coupling to diUB. Huwe1 plays essential roles in DNA repair, cell cycle regulation and development of nervous systems with its HECT domain capable of synthesizing K48-linked UB chains among other chain types,[14] while Nedd4 E3 is specific for K63 chain synthesis for signaling the endocytosis of ion channel proteins.[12c,15] To confirm the site of disulfide formation as the catalytic Cys residues of the catalytic domains of Ube2S, Huwe1 and Nedd4, we additionally mutated these Cys residues to Ala and showed that the cysteine-free protein variants were no longer reactive with the diUB probes of the designated linkages (Figure S4).

Dethiolation of the diUB conjugate with the G76C mutation installs a Dha functionality that can trap DUBs with a nucleophilic Cys residue. We set up the dethiolation of K48-diUB by reacting it with DBHDA following a reported procedure (Scheme 1).[16] We tested the reactivities of the Dha-functionalized diUB probe with the K48 linkage with the DUBs OTUB1, OTUB2 and USP2.[17] We found the DUBs could form covalent adducts with the diUB probe, albeit to different degrees. The amount of adduct formation generally increased with reaction time (Figure 3). As a control, we pretreated the Dha76-diUB probe with β-mercaptoethanol to quench the reactivity of diUB

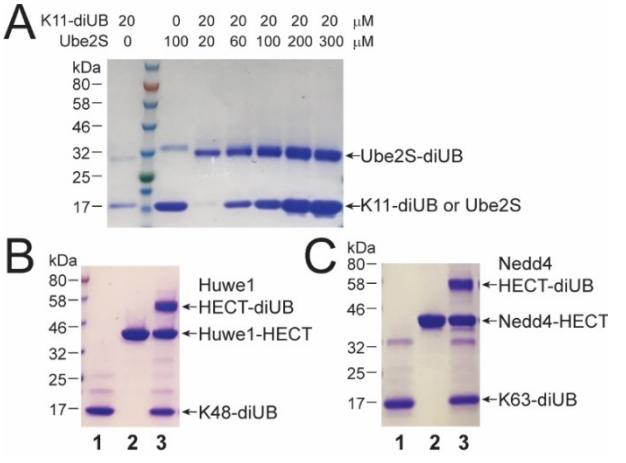

Figure 2. Synthesis of diUB conjugates with E2 and HECT E3s. (A) Formation of the disulfide conjugates between the catalytic domain of Ube2S (C118A) and K11-diUB. Varying concentrations of Ube2S were used for the coupling reactions with K11-diUB. (B) Formation of disulfide conjugates between a single-cysteine variant of the Huwe1 HECT domain and K48-diUB. 1, diUB only; 2, HECT only; 3, coupling reaction. (C) Formation of disulfide conjugates between a single-cysteine variant of the Nedd4 HECT domain and K63 diUB. Same lane assignment as in (B).

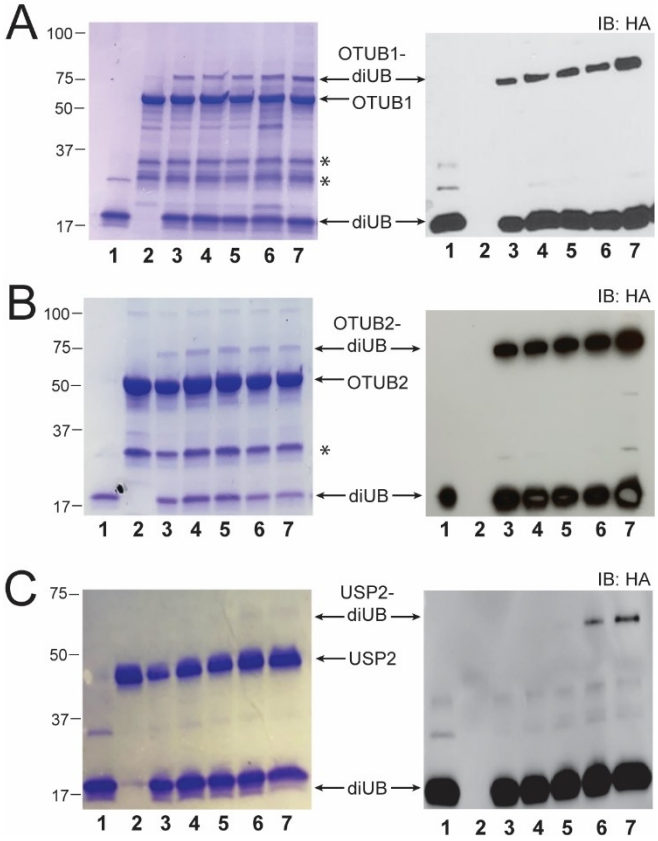

Figure 3. Reactivity of DUBs with diUB probe of K48 linkage with the G76Dha functionality at the diUB linkage. The diUB probe was reacted with OTUB1 (A), OTUB2 (B) and USP2 (C) for varying lengths of time. 1, diUB only; 2, DUB only; 3, reaction for 0.5 hour; 4, 1 hour; 5, 2 hours; 6, 4 hours; 7, 6 hours. The reactions were analyzed by SDS-PAGE (left panels) and Western blotting probed with an anti-HA antibody that would recognize the HA tag on the diUB probe. * designates protein contaminants copurified with OTUB1 and OTUB2 from *E coli*.

by capping the Dha group. Indeed, we found the pretreated diUB probes are no longer reactive with the DUBs, demonstrating the dependence of the diUB probe on the Dha group for conjugation to the Cys thiol of the DUBs (Figure S5). Overall, these results suggest that the generated diUB conjugates can be used as activity-based probes of DUBs.

This study developed a method for linkage-specific synthesis of diUB based on the replacement of a designated Lys in the acceptor UB with ThzK UAA and conversion of the ThzK residue to CysK to facilitate site-specific ligation with the thioester form of the donor UB (Scheme 1). A recent report showed the incorporation of ThzK into UB followed by its conversion to CysK and reaction with the donor UB with a C-terminal aldehyde to generate a diUB with a thiazolidine ring linkage.[6d] We chose to carry out expressed protein ligation between CysK UB and UB~SR so the diUB product would have a native isopeptide linkage except for a G76C mutation at the junction. The Cys residue installed by the UAA mutagenesis could then be conjugated to the catalytic Cys residues of E2 and E3 enzymes by disulfide formation or be transformed into a Dha electrophile for trapping DUBs. diUB with a Dha functionality has been produced by dethiolation of linkage specific G76C diUB prepared by solid-phase peptide synthesis.[16,18] Here we demonstrate that we can prepare the diUB probes by UAA incorporation, providing facile access of these reagents for probing the function of DUBs. Following the same strategy, we envision we can take advantage of UAA incorporation to prepare UB-substrate or UB-E2 conjugates of defined linkages that were previously produced by total protein synthesis for installing the Dha functionality.[19] diUB and triUB probes with a Dha trap have also been generated by mutating specific Lys residues on UB to Cys followed by coupling with a donor UB through an α-bromovinylketone moiety.[20] UAA mutagenesis adds another powerful tool for generating designer UB conjugates to probe the chemical mechanism of UB transfer and decipher the biological signals carried by protein ubiquitination in the cell.

Experimental Section

Details of the experimental procedures are provided in the Supporting Information.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: expressed protein ligation · deubiquitinating enzymes · reactivity-based probes · ubiquitin · unnatural amino acids

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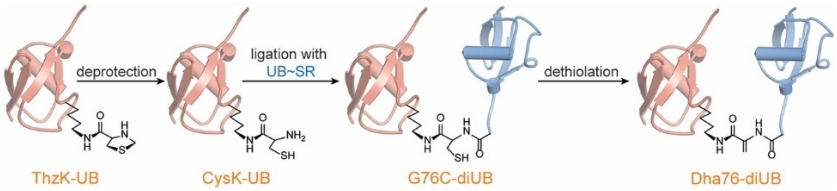

Unnatural amino acid replacement of designated Lys residues in ubiquitin facilitates the synthesis of linkage-specific di-ubiquitin probes that can capture enzymes for ubiquitin transfer and cleavage.

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Linkage-Specific Synthesis of Di-ubiquitin Probes Enabled by the Incorporation of Unnatural Amino Acid ThzK