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Ubiquitin chains bearing genetically encoded photo-crosslinkers enable efficient covalent capture of (poly)ubiquitin-binding domains.

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Abstract

Ubiquitin-mediated signaling pathways regulate essentially every aspect of cell biology in eukaryotes. Ubiquitin receptors typically contain ubiquitin-binding domains (UBDs) that have the ability to recognize monomeric ubiquitin (Ub) and polymeric Ub (polyUb) chains. However, how signaling specificity is achieved remains poorly understood, and many of the UBDs that selectively recognize polyUb chains of particular linkages still need to be identified and characterized. Here we report the incorporation of a genetically encoded photo-crosslinker, *p*-Benzoyl-L-phenylalanine (Bpa), into recombinant Ub and enzymatically synthesized polyUb chains. This allows photo-crosslinking (covalent bond formation) of monoUb and K48- and K63-linked diUb chains to UBDs. This approach provides a framework for understanding Ub cellular signaling through the capture and identification of (poly)Ub-binding proteins.

Graphical Abstract

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Supporting Information

Experimental methods and supporting figures and tables are found in the Supporting Information. The Supporting Information is available free of charge on the ACS Publications website.

Keywords

Ubiquitin; Photo-crosslinking; Ubiquitin Binding Domains; *p*-benzoyl-L-phenylalanine

Ubiquitin (Ub) is a small protein that serves as a posttranslational modification (PTM) in eukaryotes that controls essentially all cellular processes, including protein turnover and trafficking, DNA repair, and signal transduction. This is achieved through ubiquitination, the covalent conjugation of Ub's C-terminal glycine residue (G76) to the ϵ -amino group of a lysine residue on a substrate protein.^{1–5} Ubiquitination is mediated through a three-step enzymatic cascade, consisting of an E1 activating enzyme (E1), conjugating enzyme (E2), and ligase (E3). Ubiquitin modifications can include single or multiple target lysines tagged with either monomeric Ub (monoUb) or polymeric Ub chains (polyUb). Importantly, Ub contains seven lysine residues (K6, K11, K27, K29, K33, K48, and K63), each of which can serve as a polymerization site, resulting in a wide range of potential polyUb tags/signals. It has been suggested that the topology of these different linkages can give rise to different polyUb conformations and different functional activities within cells.⁶ For example, K48-linked polyUb chains signal for proteasomal degradation of tagged proteins, while K63-linked polyUb chains are involved in regulating DNA repair and signal transduction.^{6,7} Significant unanswered questions remain regarding the connection between the polyUb signal and its biological effect, namely which cellular receptors/binding partners mediate the various biological outcomes. Finding these requires technology that can capture transient interactions with specificity.

Ubiquitin-binding domains (UBDs) are small modular protein domains that bind non-covalently to Ub, and can mediate protein-protein interactions in Ub-signaling pathways. Much of this recognition involves a hydrophobic surface patch comprising Ub residues L8, I44, and V70.^{6–8} Previous studies have identified numerous UBDs for monoUb and polyUb chains through bioinformatics, yeast two-hybrid screens, and affinity pull-down technology.⁹ UBDs for monoUb and polyUb typically exhibit moderate to low affinity binding, ranging from ca. 3 μ M to 2 mM. To enhance the understanding of specific polyUb recognition by UBDs and capture low-affinity binding, photo-activatable crosslinkers have been incorporated into polyUb chains, in which photo-leucine containing Ub monomers were produced using linear total chemical synthesis.¹⁰ Total chemical synthesis has also been used to incorporate a diazirine photo-crosslinker into K48-linked and K63-linked diUb chains.¹¹ As an alternative strategy, genetically encoded crosslinking amino acids *p*-benzoylphenylalanine (Bpa) and *p*-azidophenylalanine (AzF) have been incorporated into the monomeric small ubiquitin-like modifier (SUMO).¹² These studies used photo-crosslinkers to covalently bond to known UBDs or peptide sequences and proteins from cellular lysates.

To investigate whether genetically encoded crosslinkers are a viable strategy for capturing weak or transient polyUb-target interactions, we chose to work with the ubiquitin-associated domain 2 (UBA2) of the human homolog of budding yeast Rad23 protein (hHR23a)^{13,14} and tandem ubiquitin-interacting motifs (tUIM) of human Rap80¹⁵ that were shown to have binding specificity for K48- and K63-linked Ub chains, respectively. Here we have chosen to

incorporate the chemically stable *p*-benzoyl-L-phenylalanine (Bpa) into Ub at selected sites using bacterial expression (Figure 1A). Our approach was to perform genetic encoding of Bpa using an orthogonal suppressor tyrosyl tRNA (tRNA^{Tyr}) and *Methanococcus jannaschii* tyrosyl-tRNA synthetase (MjTyRS) pair.¹⁶ The codons corresponding to the desired positions in Ub were mutated to the amber stop codon, TAG, for Bpa incorporation. Positions for mutation to Bpa were selected based on the three-dimensional structure of Ub (Figure 1B). Residue positions were chosen that either belong to or are adjacent to the hydrophobic surface patch of Ub, a critical Ub-recognition element.⁸ These include residues L8, T9, G10, K11, T12, S20, R42, A46, K48, Q49, E51, D52, H68, and V70. Residues S20 and D52 serve as negative controls as they are not located close to the potential binding interface, and therefore are positions in which crosslinking should not occur. Each mutant was expressed and purified to homogeneity in good yields as a C-terminal hexahistidine-tagged protein (His₆-tag) (Supporting Information (SI), Fig. S1).

To identify positions that undergo crosslinking, we tested monomeric Ub variants containing single-site Bpa mutations with UBA2 domain. The proteins were mixed on ice and half of the sample reaction subjected to irradiation with a stationary UV lamp (365 nm) for 30 min. Samples were analyzed initially by Coomassie-stained SDS-PAGE, however, no significant crosslinking was observed (data not shown). The binding affinity of UBA2 for mono-Ub is weak, with a K_d of 400–500 μ M,^{17–19} and we therefore thought there may be crosslinking, but at yields below the detectable limits of a Coomassie stain. These samples were further analyzed using silver-stained gels which revealed potential crosslinked proteins formed, albeit at very low abundance (<1%) (SI, Figs. S2 and S3). Interestingly, the band intensity of the crosslinked protein varied depending on the site of Bpa incorporation, with the strongest band intensity occurring in the instances where the Bpa residue was located at positions 8, 9, 42, 48, 49, or 52. Similar observations were made for crosslinking of Ub and the UBA domain of human ubiquitin-1 protein (SI, Figs. S2 and S3, Table S1). These results indicate that crosslinking efficiency depends on the site of Bpa incorporation and suggest that the abovementioned sites are the most reactive positions for capturing transient inter-molecular interactions.

We decided to investigate covalent capture further by preparing diUb chains that contain a Bpa at position 9 in the proximal Ub unit. The structural integrity of this variant was verified by NMR (SI, Fig. S4), which shows little variance from that of wild-type Ub, apart from local changes in resonances close to the site of mutation. The proximal unit in a Ub chain is the one that contains a free C-terminus, and to which the next (distal) Ub unit is covalently attached. Chains were assembled enzymatically using a chain-terminator Ub building block carrying a K48R or K63R mutation on the distal Ub and a C-terminal extension (His₆ tag) or truncation (Ub74) on the proximal Ub. This allows full control of the E2-catalyzed conjugation reaction, and enables domain-specific incorporation of mutations or isotope labeling^{18,20–22} (Figure 1C). The conjugating E2 enzymes used for the K48-linked and K63-linked dimers formation were E2–25K-GST and Ubc13-GST/Mms2-His₆, respectively.^{18,22} Of note, all the Ub variants we tested were efficiently processed into diUb chains, which indicates that a single Bpa mutation does not affect the substrate recognition characteristics by E2 enzymes. After preparation, we tested the ability of these chains to cross-link with UBA2. UBA2 has a greater affinity for K48-linked diUb chains (K_d = 8 μ M) than for K63-

linked diUb ($K_d \sim 200 \mu\text{M}$) or monoUb ($K_d \sim 400\text{--}500 \mu\text{M}$).^{14,18} Upon analysis of a Coomassie stained SDS-PAGE gel, a cross-linked band was observed for K48-linked dimers bearing a Bpa mutation at position 9, while no cross-linking was observed for diUb linked via K63 (Figure 2A,B). These cross-linking results agree with *in vitro* data on UBA2's Ub chains binding preferences. Importantly, we detected no self-crosslinking in reactions that contained diUb alone (SI, Fig. S6), which supports the notion that crosslinking will only occur for proteins interacting with Ub near the Bpa residue.

In a complementary experiment, Rap80 tUIM, which was shown to have binding preference for K63-linked diUb chains was used as the binding partner. Rap80 has a seven-fold difference in affinity for the two diUb linkages, with a $K_d = 22 \mu\text{M}$ for K63-linked, and $K_d = 157 \mu\text{M}$ for K48-linked diUbs.¹⁵ An analogous experiment using the same chains revealed a cross-linked band corresponding to Rap80-diUb. In contrast to UBA2, cross-linking was observed for both K48- and K63-linked diUbs (Figure 2C,D). We performed similar experiments using diUb chains with Bpa inserted in the proximal Ub unit at positions 11, 46, 48, or 49, all of which are summarized in Table S2. As in the case of monoUb, Bpa at positions 9 and 49 were most reactive.

The diUb experiments described above all correspond to capturing interactions when the reactive moiety is located in the proximal Ub unit in the chain. In order to confirm that UBD-capturing capability is not limited to the proximal Ub, we assembled Ub dimers bearing Bpa in the other, distal Ub unit. For this we generated Ub mutants that contain either K48R or K63R chain-terminating mutations in addition to a Bpa mutation. These double-mutant building blocks were then reacted with a wild-type Ub containing a C-terminal hexahistidine tag to form Ub dimers. Using the same enzymatic assembly strategy as above, we produced K48- and K63-linked diUbs that now contained the photo-crosslinker in the distal Ub unit. Interestingly, despite the Bpa at position 9 being in a different Ub unit/orientation relative to the binding partner, we see evidence of crosslinking ($\sim 5\%$) of both K48- and K63-linked Ub dimers to both binding partners, indicating again, that this position is very reactive (SI, Fig. S7 and Table S2). Of note, the fact that Bpa-containing Ub was activated by E1 and incorporated as the distal unit in the diUb chain further indicates that both the E1 and E2 enzymes used here tolerate Bpa insertion at position 9 in Ub. For a kinetic comparison of 9pBpa and wild-type Ub in ubiquitination reactions catalyzed by E1 and E2, see SI, Fig S8.

Finally, to test if Bpa-containing diUbs can capture significantly larger binding partners, we used a 252 amino acid fragment (residues 391–642) of the Rpn1 (Rpn1^{391–642}) subunit of the proteasome which recently has been shown to serve as a proteasomal receptor for polyUb.^{10,23} As is evident from Figure 3, both K48- and K63-linked diUbs having Bpa at position 49 in the proximal Ub were able to crosslink with Rpn1^{391–642}. This is fully consistent with published NMR data¹⁰ that showed that K48- and K63-linked diUbs have comparable binding affinities ($K_d \sim 110 \mu\text{M}$) for Rpn1^{391–642}. In addition, the presence of two crosslinking bands on the gel in Figure 3 likely reflects the fact that Rpn1^(391–642) contains two Ub-binding surfaces/sites,²³ hence possibly resulting in two distinct cross-linked configurations.

Our current findings demonstrate that Ub chains can be converted into sensitive photo-affinity probes using unnatural amino acid mutagenesis. Our results show that of all the placements of Bpa tested here, positions 8, 9, 10, 12, 42, 48, 49, 51, and 68 showed crosslinking to hHR23A UBA2, and the same positions, except for 10 and 68, gave crosslinking to ubiquitin-1 UBA. For the dimers tested, Bpa incorporated at positions 9 and 49 in the proximal Ub and 9 in the distal Ub of K48- or K63-linked diUbs was able to capture the respective binding partners (UBA2 or Rap80 tUIM, respectively). The location of the successful crosslinking sites can be seen in the SI, Figs. S9 and S10. These probes can capture weak or transient protein-protein interactions which are often critical in Ub-mediated signaling. Importantly, the Bpa moiety can be reversibly excited depending on time of irradiation, and therefore these capture processes do not necessarily match binding affinities. Rather, we view this approach as a useful method to trap potential interaction candidates (including relatively weak binders) that can be subsequently analyzed by traditional biophysical techniques. While it is expected that in some situations, Bpa incorporation could alter the affinity of these interactions, we do not view this as a problem if cross-linking still occurs. In the case of 9pBpa and 46pBpa, we performed an NMR-based binding assay with the UBA domain of human ubiquitin-1 protein (UBAy) and see very limited changes resulting from the mutation (see SI, Fig S11). Importantly, we have shown that ubiquitination cascades catalyzed by E1 and E2 enzymes can accommodate Ub with unnatural amino acid labels to produce chains containing photo-affinity probes in the distal or proximal units. These dimers are functional and can form covalent linkages with known UBDs. This strategy can readily be extended to longer Ub chains, with the photo-crosslinker incorporated at any desired Ub unit (or multiple units) in the chain. This methodology can also be applied to make Ub chains containing other linkages using enzymatic or non-enzymatic chain assembly²⁴. Furthermore, genetic encoding of photo-affinity probes into Ub chains assembled from recombinant Ub monomers provides a viable alternative to total chemical synthesis as it can be achieved using tools readily available in a biochemical laboratory setting. Finally, we have identified hotspot mutations in the context of monomeric or dimeric Ubs that are more reactive towards capturing intermolecular interactions. We also envision that it might be possible to fine tune crosslinking specificity for different binding partners by choosing different positions for probe(s) incorporation into a polyUb chain. This technology now sets the stage for finding and interrogation of unknown binding partners of polyUb chains using combinations of crosslinking in a cellular context followed by proteomics identification.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

Ub	ubiquitin
UBD	ubiquitin binding domain
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
UBA	ubiquitin-associated domain
UQ1	ubiquilin-1
UBA2	ubiquitin-associated 2 domain
hHR23a	human homolog of budding yeast Rad 23
tUIM	tandem ubiquitin-interacting motif

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Figure 1.

Photo-crosslinking methodology and structures. a) Structure of p-benzoyl-L-phenylalanine (Bpa). b) Structure of Ub (tan) showing hydrophobic patch (red) and sites chosen for Bpa incorporation (PDB: 1UBQ). c) Schematics of enzymatic assembly of diUb with photo-crosslinker, Bpa, in the proximal Ub unit and capture of a UBD upon photo-activation.

Figure 2. SDS-PAGE analysis of photo-crosslinking of diUb chains containing K48 and K63 linkages when irradiated at 365 nm. A) K48-linked diUb and B) K63-linked diUb with UBA2 domain. C) K48-linked diUb and D) K63-linked diUb with Rap80 tUIM. Bpa was inserted in the proximal Ub at position 9. The gels were Coomassie stained; the bands corresponding to crosslinked product are circled.

Figure 3. Photo-crosslinking of K48- and K63-linked diUb chains having Bpa at position 49 in the proximal Ub with Rpn1³⁹¹⁻⁶⁴², visualized using Coomassie-stained SDS-PAGE gel of the reaction.