Identification of Proteins Interacting with Ubiquitin Chains

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Abstract: Ubiquitylation, the modification of proteins with ubiquitin (Ub), is one of the most versatile post-translational modifications in eukaryotic cells. Since Ub also serves as its own substrate, proteins can be modified by numerous different Ub chains, in which the individual moieties are linked via one or several of the seven lysines of Ub. Homogeneous Ub chains, in which the moieties are sequentially linked via the same residue, have been most extensively studied. However, due to their restricted availability, the functions of Ub chains linked via K27, K29, or K33 are poorly understood. We have developed an approach that, for the first time, allows the generation of all seven homogeneous Ub chains in large quantities. The potential of our approach is demonstrated by the identification of previously unknown interaction partners of K27-, K29-, and K33-linked Ub chains by affinity-based proteomics.

Ubiquitylation is mediated by a complex enzymatic cascade and results in the formation of different conjugates. In its most simple form, one Ub moiety is attached to substrate proteins through the formation of an isopeptide bond between the carboxylate of the C-terminal glycine of Ub and the ε-NH₂-group of a lysine in the substrate. Ub contains seven lysines (K6, K11, K27, K29, K33, K48, K63) and each of these, as well as the N-terminal α-NH₂ group, can be ubiquitylated. Thus, substrate proteins can be modified with K-linked Ub chains (i.e., Ub moieties are connected via lysine residues by isopeptide bond formation) or in some cases with K-linked Ub chains (i.e., Ub moieties are linked via one lysine residue, can be hydrolyzed by DUBs, and since eukaryotic cells are rich in DUBs, the applicability of these Ub chains for the identification of chain-selective interaction partners is limited. Therefore, we and others have developed approaches to generate DUB-resistant triazole-linked chains and reported the synthesis of four linkage-specific chains (K11, K27, K29, K48). However, expression and purification of the monomer precursors were cumbersome, since two unnatural amino acids (UAAs) had to be incorporated into the same Ub in a cell-based system, which not only lowered the yields of full-length Ub but also resulted in the formation of truncated forms of Ub. Stanley and Virdee, and Brik and co-workers have described a similar concept for the generation of K6-linked Ub chains and Ub-protein conjugates, respectively, through oxime ligation. However, so far none of the developed methods has been shown to enable the generation of all seven homogeneous chains in sufficient yields or the discovery of chain-selective interaction partners.

Here, we present a facile and robust approach for the preparation of all seven Ub chains (up to ten Ub moieties) by isopeptide bond mimicry in milligram quantities. The approach combines the incorporation of a single UAA into the same (homogeneous chains), different (heterogeneous chains), or two or more (branched chains) lysine residues per Ub moiety. The theoretically infinite number of different chains and the notion that different Ub chains may direct modified proteins to different fates make ubiquitylation one of the most complex and versatile regulators of biological processes.

Homogeneous Ub chains are the most extensively studied chain types. K48-linked chains, and presumably K11-linked chains, target substrates for proteasomal degradation, while K63-linked and linear chains regulate the function of proteins in a non-proteolytic manner, for example, in signal transduction pathways and DNA repair. The physiological functions of other chain types (e.g., K27, K29, K33) are less well understood, which is in part due to the fact that only very few linkage-selective interaction partners are known so far.

A difficulty in studying K-linked Ub chains is to obtain these in sufficient amounts and lengths. For instance, the enzymatic assembly of K27-linked chains is still not feasible due to a lack of corresponding conjugating enzymes. Much effort has been made to synthesize Ub chains through protein polymerization. Brik and co-workers have utilized solid-phase peptide synthesis combined with native chemical ligation to assemble K48- or K63-linked chains. Strietter and co-workers have employed thiol–ene coupling to synthesize homogeneous Ub chains linked at K6, K11 or K63. These isopeptide-linked or thioether-linked Ub polymers were utilized to study the selectivity of de-ubiquitylating enzymes (DUBs) for distinct Ub–Ub linkages. However, since they can be hydrolyzed by DUBs, and since eukaryotic cells are rich in DUBs, the applicability of these Ub chains for the identification of chain-selective interaction partners is limited. Therefore, we and others have developed approaches to generate DUB-resistant triazole-linked chains and reported the synthesis of four linkage-specific chains (K11, K27, K29, K48). However, expression and purification of the monomer precursors were cumbersome, since two unnatural amino acids (UAAs) had to be incorporated into the same Ub in a cell-based system, which not only lowered the yields of full-length Ub but also resulted in the formation of truncated forms of Ub. Stanley and Virdee, and Brik and co-workers have described a similar concept for the generation of K6-linked Ub chains and Ub-protein conjugates, respectively, through oxime ligation. However, so far none of the developed methods has been shown to enable the generation of all seven homogeneous chains in sufficient yields or the discovery of chain-selective interaction partners.

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Here, we present a facile and robust approach for the preparation of all seven Ub chains (up to ten Ub moieties) by isopeptide bond mimicry in milligram quantities. The approach combines the incorporation of a single UAA into
Ub by selective pressure incorporation (SPI) with Michael addition and copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC) reactions. By using such DUB-resistant polymers in a mass spectrometry (MS)-based affinity enrichment assay, we identified several proteins that interact with K27-, K29-, and/or K33-linked chains, thus demonstrating the potential of our approach to elucidate the physiological functions of Ub chains in general and of so far poorly characterized Ub chains in particular.

To functionalize Ub with an azide at its C-terminus, we constructed a Ub cDNA in which the Gly76 codon was deleted and Gly75 was replaced with a codon for methionine except for the initial one, a fusion protein system was used consisting of Ub lacking the initial Met, fused to the C terminus of glutathione-S-transferase (GST). This resulted in the later generated triazole linkage. To avoid incorporation of Aha at the start codon of Ub (note that wild-type Ub does not harbor any methionine except for the initial one), a fusion protein system was used consisting of Ub lacking the initial Met, fused to the C terminus of glutathione-S-transferase (GST). In this way, the later generated triazole linkage has a similar number of atoms as the native isopeptide linkage. To enable the incorporation of azidohomoalanine (Aha) by SPI (Figure 1A). In this way, the later generated triazole linkage was used as an additional control. After incubation with HEK293T cell lysate (note that the triazole-linked Ub chains cannot be hydrolyzed by DUBs and thus stay intact in cell lysate; Figure S5) and thorough washing, the Ub polymers and proteins bound to these were eluted with biotin. The elution fractions were resolved by SDS-PAGE (Figure S6) and subsequently analyzed by LC–MS/MS and label-free quantification.

Since our aim was to identify interaction partners of the three different Ub chains, significant enrichment of potential binding proteins was determined with respect to proteins found in the mono-Ub sample (Figures 2B–D; for a full list see Table S1). The Venn diagram in Figure 2E provides an overview on how many of the enriched proteins were specific for one linkage type and how many of these were found to interact with different linkage types. For K27-linked Ub chains, we were able to identify 70 interaction partners. For K29- and K33-linked chains, we identified 44 and 37 proteins, respectively. A majority of 72.8% (51 out of 70) of the interaction partners with K27-linked chains were unique for this linkage type. For chains linked via K29 and K33, the selectivity was lower: 31.8% (14 out of 44) and 24.3% (9 out of 37), respectively. K29- and K33-linked chains also showed a relative strong overlap in interaction partners (24 out of 44 for K29-linked chains and 24 out of 37 for K33-linked chains), thus suggesting similar biological functions. Importantly, the proteins that were selectively enriched in the mono-Ub samples showed a high degree of consistency for all 3 investigated linkage types (Table S1), thus corroborating the reproducibility and robustness of our approach.

Next, we confirmed the interaction of some of the identified proteins with the respective chain type by western blot analysis (Figure 3). The obtained results were in excellent agreement with our proteomic profiling data. Of special interest were the proteins identified as selective interaction partners of K27-linked Ub chains, since this linkage type has the least information available among the homogeneously linked chains. We confirmed the interactions by western blot analysis for all three tested proteins: PLAA, RANBP9 and AIFM1 (Figure 3).
The yeast orthologue of PLAA (Ufd3/Doa1) harbors a Ub-binding domain (Pfu domain) and interacts with Cdc48 (mammalian p97/VCP), an ATPase involved in several Ub-dependent pathways.\[14\] Mammalian PLAA is less well studied, but like Doa1 it has been connected to the endolysosomal system.\[15\] Gid1, the yeast homologue of RANBP9, is part of a conserved E3 ligase complex involved in the degradation of gluconeogenic enzymes.\[16\] Whether this is also the case for RANBP9 remains to be shown. AIFM1 is mainly involved in mitochondrial processes, including induction of apoptosis.\[17\] Notably, K27-linked Ub chains have already been linked to mitochondrial processes.\[18\]

We also confirmed the binding of ZRANB1, WRNIP1, USP13, and TOM1, which according to our MS measurements selectively interact with K29- and K33-linked chains, by western blot analysis (Figure 3). The DUB ZRANB1 is the only so far known interaction partner for K29- and K33-linked chains,\[19\] while WRNIP1 is a DNA repair protein containing a UBZ domain that binds to Ub chains.\[20\] The DUB USP13 has been shown to be involved in various processes such as autophagy and endoplasmic reticulum associated degradation (ERAD).\[21\] TOM1 has been suggested to be involved in the endosomal trafficking of ubiquitylated proteins, but no preference for a certain linkage type has been reported so far.\[22\]

Finally, the interaction of ZRANB1, WRNIP, USP13, and TOM1 with K29- and K33-linked chains was confirmed by competition experiments using the respective isopeptide-linked Ub tetramers (Figure S7). Indeed, addition of K29- and K33-linked Ub tetramers efficiently competed with the binding of ZRANB1, WRNIP, USP13, and TOM1 to the respective triazole-linked Ub chains, while binding of PLAA to K27-linked chains was not affected. This not only shows that the proteins identified bind to the Ub chains studied in a linkage-selective manner (i.e. K29- and K33-linked chains cannot compete with K27-linked chains for PLAA binding, while ZRANB1, WRNIP, USP13, and TOM1 are bound by...
K29- and K33-linked chains but not by K27-linked chains) but also strongly indicates that the triazole-linked Ub chains resemble native isopeptide-linked Ub chains at the structural and functional level.

Recently, Zhang et al. published a comprehensive study on the interaction partners of Ub dimers, in which the Ub moieties were connected by a triazole linkage that is structurally different to the one introduced here. Comparison of the data shows that five out of the seven proteins (PLAA, ZRANB1, WRNIP1, USP13, TOM1) studied here were also identified by Zhang et al., with some of these (PLAA, WRNIP1, USP13) not showing much preference in their binding behavior to differently linked Ub dimers. Although we identified them as interaction partners that discriminate between K27-linked (PLAA) and K29- and K33-linked (WRNIP1, USP13) Ub chains, they may in addition have the potential to bind to Ub chains linked via residues other than K27 or K29/K33. Furthermore, two proteins identified here as interaction partners of K27-linked chains (RANBP9, AIFM1) were not identified by Zhang et al. at all. Since Ub polymers longer than Ub dimers were employed in our study, this data supports the notion that linkage-selective protein interactions are affected by the length of the Ub chains. Our approach for generating linkage-specific Ub chains thus significantly expands the toolbox for studying the complex mechanisms of Ub signaling.

The chains bear a significant degree of polymerization (up to 10 Ub moieties) and are stable in cell lysates. This enabled us to identify interaction partners of the three poorly characterized linkage types K27, K29, and K33 through affinity-based proteomic profiling. Importantly, several of the interactions identified by MS were confirmed by western blot analysis, thus demonstrating the potential of our approach. The presented method provides an important tool to unravel the complex mechanisms of Ub signaling.

Acknowledgements

This work was supported by the DFG (SFB969, project B3). X.Z. and E.H. thank the DAAD and the Konstanz Research School Chemical Biology, respectively, for support by a fellowship. E.S. acknowledges funding from the DFG Emmy Noether Program (STE 2517/1-1). We further thank Dr. Samra Ludmann for graphical support.

Conflict of interest

The authors declare no conflict of interest.

Keywords: affinity-based profiling · click chemistry · post-translational modification · proteomic profiling · ubiquitylation
