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The linkage specificity determination of Ube2g2-gp78 mediated polyubiquitination

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ABSTRACT

Polyubiquitin chain linkage specificity or topology is essential for its role in diverse cellular processes. Previous studies pay more attentions to the linkage specificity of the first ubiquitin moieties, whereas, little is known about the editing mechanism of linkage specificity in longer polyubiquitin chains. gp78 and its cognate E2-Ube2g2 catalyze lysine48 (K48)-linked polyubiquitin chains to promote the degradation of targeted proteins. Here, we show that the linkage specificity of the entire polyubiquitin chain is determined by the conjugation manner of the first ubiquitin molecule but not the following ones. Further study discovered that the gp78 CUE domain works as a proofreading machine during the growth of K48-linked polyubiquitin chains to ensure the linkage specificity. Together, our studies uncover a novel mechanism underlying the linkage specificity determination of longer polyubiquitin chains.

1. Introduction

Polyubiquitination is a kind of well characterized protein post-translational modification which could alter protein localization, activity, stability etc [1]. Polyubiquitin chains were assembled via isopeptide linkages between the ε-NH_2 group on lysines (K) of the acceptor ubiquitin and the C terminus of the donor ubiquitin. Any of the seven lysines (K6, K11, K27, K29, K33, K48, K63) on the acceptor ubiquitin could be used for polyubiquitin chain assembly, thus each linkage gives the polyubiquitin chain a distinct topology [2]. In addition, there also exist linear ubiquitin chains as the amino terminus of a substrate-linked ubiquitin can serve as an acceptor for the coming ubiquitin molecule [3]. A mixture of different linkages could form branched ubiquitin chains, which may change the final destiny of the existing chains [4]. Polyubiquitin chains of different linkages exist in cells at varying levels of abundance, each of them drives their targeted cargos to a certain destination [5].

Ubiquitin chain linkages have been recognized as cellular signals conducting various biological processes. For example, proteins conjugated with K48-linked polyubiquitin chains were delivered to the 26S proteasome for degradation [6], whereas those conjugated with K63-linked chains changed their localization or interaction proteins [7], and linear ubiquitin chains recognized by NEMO were found to be important for NF-κB activation [8]. Despite the essential role of polyubiquitin chains and their topology, little is known about the mechanism underlying their linkage specificity determination.

It is well understood that ubiquitin chains were assembled by a series of enzymes sequentially: an activating enzyme (E1), a conjugating enzyme (E2), and a ubiquitin ligase (E3) [9]. Linkage specific chains were always synthesized by certain E2-E3 pairs, for example, Ube2S-APC/C synthesis K11-linked ubiquitin chains [10], Ube2g2-gp78 synthesis K48-linked ubiquitin chains [11]. Previously, much more attentions had been paid to the linkage of the first two ubiquitins in the ubiquitination processing, and these findings help us understand the mechanism underlining the chain initiation and linkage establishment. The subunit of Ube2S orients the donor ubiquitin to bind only the acceptor surface around Lys11, thus forming a catalytically competent active site composed of residues of both Ube2S and ubiquitin [12]. This unique orientation and active site allows the donor ubiquitin to attack only the lysine11, and form K11-linked ubiquitin chains. gp78 could assemble preformed K48-linked ubiquitin chains on Ube2g2 [13], and in this system, Ube2g2 could

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form homodimer, then the dimeric Ube2g2 catalyzed donor and acceptor ubiquitins to form K48-linked ubiquitin chain. In detail, a highly conserved arginine residue in the donor Ube2g2 senses the presence of an aspartate in the acceptor ubiquitin to position only Lysine48 of ubiquitin in proximity to the donor E2 active site [14]. In addition, we found that gp78 could build ubiquitin chain from the distal end by a sequential addition mode [15]. As for the linkage determination of longer polyubiquitin chains in this system is still totally unknown.

In the current study, using the Ube2g2-gp78-mediated *in vitro* poly-ubiquitination system, we demonstrated that the poly-ubiquitin chain linkage specificity was determined by the first linkage of the ubiquitin chains. As mentioned above, gp78 could initiate the assembly of K48-linked ubiquitin chain, and elongate it in a K48-linked manner, and we found that the linkage of the following chains was determined by the formerly synthesized chains. On the basis of non-K48 linked ubiquitin oligomers, gp78 could build non-K48 linked polyubiquitin chains. Further investigations revealed that the gp78 CUE domain could serve as a proofreading machine in the K48-linked-polyubiquitin-assembly. The current data together with our previously reported results uncover a precise mechanism for polyubiquitin chain initiation, elongation and linkage determination.

2. Materials and methods

2.1. Antibodies and proteins

The FLAG antibody was purchased from Abmart. The Lys29-linked Ub oligomers, Lys48-linked Ub oligomers and Lys63-linked Ub oligomers were purchased from Boston Biochem (Cambridge, MA, USA).

2.2. Plasmids, strains and cell lines

The pET28-Ube2g2, pGEX-UbE1, pGEX-gp78c and its mutants, pET28-FLAG-UbK0, K6R, K11R, K27R, K29R, K33R, K48R and K63R plasmids were described previously [13]. All of the plasmids were amplified in *Escherichia Coli* Top10 cells, and the recombinant proteins were expressed in *E. Coli* BL 21 or Rosetta 2 cells.

2.3. Protein purification

F^UbK0, K6R, K11R, K27R, K29R, K33R, K48R and K63R, Ube2g2, and gp78c were purified as previously described [11]. The purified E2 and E3 variants were further fractionated by size exclusion chromatography on Superdex75 and Superose 6 columns, respectively, in a buffer containing 50 mM Tris–HCl, pH 8.0, 150 mM potassium chloride, 5% glycerol, and 2 mM magnesium chloride.

2.4. Single-round ubiquitin transfer assays

To detect the transfer of FLAG tagged ubiquitin variants by gp78 or its variants, Ube2g2 (300 nM) was incubated with E1 (60 nM), together with either FLAG tagged ubiquitin variants (as donor) or Ub oligomers (K48, K29 or K63-linked oligomers, as acceptor), at 37 °C for 15 min in reaction buffer (25 mM Tris–HCl, pH7.4, 2 mM magnesium/ATP, and 0.1 mM DTT). The reaction was treated with 50 mM EDTA and 10 mM NEM (N-Ethylmaleimide) at room temperature for 15 min. Equal volumes of the donor and acceptor were mixed into the reaction and catalyzed by GST-gp78c or its variants at 37 °C for indicated duration. The reaction was stopped by the addition of Laemmli buffer and analyzed by immunoblotting with anti-FLAG antibody.

3. Results and discussion

3.1. Non-K48-linked ubiquitin chains could be built on preformed non-K48-linked ubiquitin oligomers

Since Ube2g2-gp78 could assemble pure K48-linked poly-ubiquitin chains on the catalyzing cysteine of the E2 [11], this *in vitro* ubiquitination system provides a nice chance for us to investigate the linkage specificity determination mechanism of longer polyubiquitin chains. To that end, a single-round ubiquitin transfer assay modified from a previous report ([15] Fig. 1A) was used. Briefly, a FLAG-tagged Ub K48R (F^UbK48R) mutant or ubiquitin oligomers were charged onto Ube2g2, and the reactions were quenched to block recharging of Ube2g2 by adding EDTA and NEM [13]. Because the Lys48 has been mutated to Arginine, this E2-ubiquitin complex could only serve as potential donor in the transfer reaction. Upon incubating these complexes with Ube2g2 precharged with an untagged wild-type ubiquitin oligomers of different length (E2~K48-linked Ub1-3) as acceptor, the transfer of F^UbK48R to the ubiquitin oligomers should then lead to the extension of the chains by one ubiquitin molecule, and the products could be detected by immunoblotting with the anti-FLAG antibody. As shown in Fig. 1B, in the presence of gp78, all the precharged K48-linked ubiquitin oligomers can only be elongated by one UbK48R molecule, and no polyubiquitin chains could be detected. These results confirmed that gp78 could only transfer one E2 charged UbK48R to the existing K48-linked oligomers, and for the lack of available Lys48 on the distal end, the newly added UbK48R ubiquitin moiety cannot serve as acceptor for the next one. These results suggested that based on the pre-assembled K48-linked ubiquitin chains, no other lysine residues on the UbK48R could be used to build polyubiquitin chains in this system.

To further test the determinate role of the existing K48 linkage, we performed the UbK48R transfer assay on the basis of di-ubiquitin oligomers of two other linkages K29 and K63 (Fig. 1C). Compare to the K48-linked ubiquitin oligomers, gp78 could elongate the K29 or K63-linked di-ubiquitins to polyubiquitin chains using UbK48R molecules (Fig. 1D line 1–3 and 1E line 1–3). If the K63-linked tri-ubiquitin oligomers were used as acceptor in this system, gp78 could also elongate the pre-assembled chains to high molecular products (Fig. 1F and G). These results demonstrate that if the pre-assembled ubiquitin oligomers were not K48-linked, then gp78 could elongate these chains via a non-K48-linked manner.

3.2. Linkage specificity is lost in the presence of preformed non-K48-linked ubiquitin chains

For gp78 could build non-K48-linked ubiquitin chains on K29 or K63-linked ubiquitin oligomers, what's the linkage of the newly built polyubiquitin chains? Whether the pre-assembled K29 or K63 linkage could determine the following chains synthesized by gp78? To answer this question, we mutated each of the available lysine residues on ubiquitin (K0, K6R, K11R, K27R, K29R, K48R or K63R). And the FLAG-tagged ubiquitin mutants were used as donors in the transfer assay, and untagged K63-linked di-ubiquitins were used as acceptors (Fig. 2A). In the presence of gp78, all of the above mutants could be transferred to K63-linked di-ubiquitins and even grown to polyubiquitin chains except K0, K48R and K63R (Fig. 2B). These results suggest that the newly built polyubiquitin chains on K63-linked di-ubiquitins were not sole K63-linked, inferring that gp78 could not guarantee a specific linkage based on non-K48-linked ubiquitin chains. To further test this hypothesis, we performed the similar experiment using K29-linked di-ubiquitin oligomers