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Mode of substrate recognition by the Josephin domain of ataxin-3, which has an endo-type deubiquitinase activity

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ABSTRACT

Ataxin-3, which is encoded by a gene that has been associated with Machado–Joseph disease, contains a catalytic N-terminal Josephin domain with deubiquitinase activity. Here, we show that the Josephin domain of ataxin 3 catalyzes endo-type cleavage of Lys48-linked polyubiquitin. Furthermore, NMR data obtained following site-specific paramagnetic spin labeling of Lys48-linked di-ubiquitin revealed that both ubiquitin units interact with the Josephin domain, with the C-terminal Gly76 of the proximal unit being situated in the vicinity of the catalytic triad of Josephin domain. Our results help to elucidate how the substrate is recognized by the Josephin domain and properly positioned for an endo-type deubiquitination reaction.

1. Introduction

Ubiquitination is a reversible protein modification that regulates various biological processes including cell-cycle progression, DNA repair, inflammatory response and cell survival [1,2]. The covalent attachment of ubiquitin (Ub) to target proteins is catalyzed by the sequential action of the Ub-activating enzyme E1, Ub-conjugating enzyme E2, and Ub-protein ligase E3. The carboxy terminus of Ub can also be attached to another Ub (termed the distal and proximal Ub moieties, respectively) through all seven lysine residues at positions 6, 11, 27, 29, 33, 48, and 63 and the amino terminus, giving rise to various types of polyUb chains [3,4], which act as fate determinants of ubiquitinated proteins. The K48-linked polyUb chain (K48-Ub) serves as a signal for protein degradation by 26S proteasomes, whereas the K63-linked polyUb chain (K63-Ub) plays a non-degradative role such as DNA repair and transcriptional regulation.

Ubiquitination is counteracted by a group of hydrolytic enzymes collectively termed deubiquitinases (DUBs), which remove Ub from protein adducts [5]. DUBs are classified into zinc metalloproteases and cysteine proteases. While the former consist of only one family, JAB1/MPN/MOV34 (JAMM) metalloproteases, the latter are further categorized into four families on the basis of the structure of the catalytic domain: Ub carboxyl-terminal

Abbreviations: DTT, dithiothreitol; DUB, deubiquitinase; K48-Ub, K48-linked polyubiquitin; K63-Ub, K63-linked polyubiquitin; MTSL, (1-oxy-2,2,5,5-tetramethyl-D-pyrroline-3-methyl)-methanethiosulfonate; OTU, ovarian tumor protease; PRE, paramagnetic relaxation enhancement; Ub, ubiquitin; UCH, ubiquitin carboxyl-terminal hydrolase; UIM, ubiquitin-interacting motif; USP, ubiquitin-specific protease

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hydrolases (UCHs), Ub-specific proteases (USPs), ovarian tumor proteases (OTUs), and the Josephin domain proteases.

One of the best-studied members of the Josephin family is ataxin-3, which possesses an N-terminal Josephin domain, two Ub-interacting motifs (UIMs), and a polyglutamine (polyQ) tract followed by the third UIM depending on splice variants [6,7]. This protein is encoded by the gene responsible for spinocerebellar ataxia type 3, also known as Machado–Joseph disease, which is an autosomal dominant neurodegenerative disease caused by expansion of the polyQ tract [8]. It has been reported that full-length ataxin-3 preferentially cleaves K63-linkage, especially that are situated in K48/K63-mixed linkage of polyUb chains with four or more Ub moieties [7,9]. Intriguingly, the intrinsic DUB activity of ataxin-3's isolated Josephin domain is directed towards K48-Ub in comparison with K63-Ub [10]. Similarly, UIM-mutated full-length ataxin-3 showed an increased activity toward K48-Ub, suggesting that the UIM motifs were involved in specific interaction with K63-Ub chains [9].

To date, several 3D structures of the Josephin domain of ataxin-3 have been determined, uncovering different conformational states [11,12]. These data suggest that the domain is in a dynamic equilibrium between open and closed conformations. Recently, NMR spectroscopic data proposed the interaction mode between the Josephin domain and K48-Ub_2 with two Ub-binding sites (S1 and S2) [10,13]. Herein, the S-site numbering is based on the standard protease nomenclature; hydrolysis occurs between the distal S1 and proximal S1′ site. Crystallographic data of the Josephin domain of ataxin-3-like protein (ATXN3L) proposed a similar interaction mode of Josephin−Ub (S1) complex compared to the NMR data [14].

It has been shown that ubiquitination of ataxin-3 directly enhances its DUB activity in mammalian cells [15] and *Drosophila* [16]. The primary ubiquitination site was identified at Lys117, which is located near the catalytic triad of the Josephin domain. Although the up-regulation mechanism by this ubiquitination remains to be elucidated, the Ub moiety at Lys117 would clearly hamper accommodation of the proximal Ub unit at the S1 site based on the previously proposed 3D models of Josephin−Ub (S1) complex [10,13].

In Ub chain disassembly, DUB-catalyzed cleavage can take place from the end (exo-type) or within a chain (endo-type). USP14, one of the proteasome-associated DUBs, exhibited an exo-type activity toward K48-Ub from the distal end [17]. By contrast, the USP domain of cylindromatosis (CYLD) and several OTU DUBs such as OTUD2 have endo-type DUB activities toward Lys63- and Lys11-linkages of heterotypic tetra- and pentaUb chains, respectively [18,19]. However, such well-defined information for DUB specificities has been very limited. It has also reported that ataxin-3 possesses an endo-type DUB activity with heterotypic polyUb containing K63 linkage flanked by K48 linkages [9]. However, it remains unknown whether this enzyme have endo-type DUB activity toward homotypic polyUb chains.

In this study, we revisit the interaction mode between the Josephin domain of ataxin-3 and K48-Ub_2. We demonstrate that the ataxin-3 Josephin domain possess an endo-DUB activity against K48-Ub and propose a distinct interaction model based on NMR data obtained using site-specific spin labeling.

2. Materials and methods

2.1. Protein expression and purification of full-length and Josephin domain of ataxin-3

The full-length human ataxin-3 was expressed in *Escherichia coli* BL21(DE3) CodonPlus (Agilent Technologies) using pHis8 vector and purified by Ni^{2+}-immobilized column. The cDNA fragment encoding the Josephin domain (residues 1–171) was inserted into the pGEX-6P-1 plasmid vector (GE Healthcare) with an N-terminal GST tag. For the expression of the Josephin domain, *E. coli* BL21 (DE3) CodonPlus transformed with the plasmid was cultured in LB or M9 minimal medium. The GST-fused protein was purified from the cell lysate on a Glutathione Sepharose 4B column (GE Healthcare). The protein was further purified using a Superdex 75 (10/30) gel filtration column (GE Healthcare). For the NMR analyses, stable isotope-labeled proteins were obtained by cultivating *E. coli* in M9 synthetic medium containing $^{15}NH_4Cl$ and $^{15}NH_4Cl/^{13}C$-glucose as the sole source of nitrogen/carbon, respectively. Stable isotope labeling of protein at selected amino acid residues were performed as previously described [20].

2.2. Deubiquitinase assay

Enzymatic synthesis of K48-Ub with hexahistidine (His_6) tag at the C-terminus of the proximal unit was performed using the protocol described in the literature [21]. Briefly, 0.2 mM Ub and 0.2 mM His_6-tagged Ub were mixed in 50 mM Tris–HCl (pH 8.0) and incubated at 310 K for 6 h in the presence of 1 μM E1, 40 μM E2–25 K, 1 mM dithiothreitol (DTT), 5 mM $MgCl_2$, 10 mM ATP, 0.6 U/ml creatine phosphokinase, 0.6 U/ml pyrophosphatase, and 10 mM creatine phosphate. After the reaction, Ub chains with the His_6 tag were purified from the reaction mixture by 100 μl of Ni Sepharose High Performance column (GE Healthcare). Deubiquitination assay using the K48-Ub substrates bound to the Ni^{2+}-immobilized beads (5 μl) was performed in a final volume of 100 μl in 20 mM Tris–HCl buffer (pH 8.0) containing 1 mM DTT at 310 K for 12 h. After the reaction, 10 μl of the resin-unbound fraction was collected, boiled in SDS-containing sample buffer, and subjected to SDS–PAGE and immunoblotted with a Ub-specific antibody (Santa Cruz Biotechnology). For the deubiquitination assay, 1 μM Josephin domain, 1 μM ataxin-3, and 2 mg/ml 26S proteasome were used.

2.3. Synthesis of K48-Ub_2 and the subunit-specific isotope and spin labeling

For NMR analysis of the ^{15}N-labeled Josephin domain, non-labeled K48-Ub_2 were synthesized in vitro as described [22]. Subunit-specific isotope labeling of K48-Ub_2 was also achieved by using ^{15}N-labeled Ub derivatives [D77 (proximal, S1) and K48R (distal, S2)]. In this paper, the Ub unit that carries the free Gly76 residues is defined as the proximal S1 Ub (which can be isopeptide-linked to a substrate through its C-terminus), whereas the distal S2 unit is that which carries the free Lys48 side chain.

For subunit-specific spin labeling, the C-terminal Gly76 of the proximal Ub or Lys48 of the distal Ub were substituted with a cysteine residue, which was disulfide-linked to a paramagnetic spin label [MTSL, (1-oxy-2,2,5,5-tetramethyl-D-pyrroline-3-methyl)-methanethiosulfonate, Toronto Research Chemicals]. Immediately before the spin labeling, DTT was removed from the buffer using desalting column (PD-10, GE Healthcare). The composition of the buffer used for equilibrium and spin labeling was 50 mM Tris–HCl (pH 8.0), 100 mM NaCl. The sulfhydryl group at the mutated site was then modified with a 10-M excess of the spin label at 310 K for 6 h. After removal of unreacted spin label reagent using PD-10 column, the spin-labeled protein was further purified using a Superdex 75 (10/30) gel filtration column.

2.4. NMR measurements and spectral analysis

NMR samples were prepared in 90% H_2O/10% D_2O (v/v), 10 mM sodium phosphate buffer at pH 7.0 and experiments were