Journal of Structural Biology xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

A conserved two-step binding for the UAF1 regulator to the USP12 deubiquitinating enzyme

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ARTICLE INFO

Article history:
Received 20 June 2016
Received in revised form 14 September 2016
Accepted 16 September 2016
Available online xxxx

Keywords:
Deubiquitinating enzyme
Allosteric regulation
Protein structure
Small angle X-ray scattering (SAXS)
Surface Plasmon Resonance (SPR)
Enzyme catalysis

ABSTRACT

Regulation of deubiquitinating enzyme (DUB) activity is an essential step for proper function of cellular ubiquitin signals. UAF1 is a WD40 repeat protein, which binds and activates three important DUBs, USP1, USP12 and USP46. Here, we report the crystal structure of the USP12-Ub/UAF1 complex at a resolution of 2.8 Å and of UAF1 at 2.3 Å. In the complex we find two potential sites for UAF1 binding, analogous to what was seen in a USP46/UAF1 complex. In line with these observed dual binding states, we show here that USP12/UAF1 complex has 1:2 stoichiometry in solution, with a two-step binding at 4 nM and 325 nM respectively. Mutagenesis studies show that the fingers sub-domain of USP12 interacts with UAF1 to form the high affinity interface. Our activation studies confirm that the high affinity binding is important for activation while the second UAF1 binding does not affect activation. Nevertheless, we show that this two step binding is conserved in the well-studied USP12 paralog, USP1. Our results highlight the interfaces essential for regulation of USP12 activity and show a conserved second binding of UAF1 which could be important for regulatory functions independent of USP12 activity.
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1. Introduction

Ubiquitination of proteins is a reversible post-translational modification that is critical for almost any cellular process. The control of these crucial pathways lies in the precise regulation of ubiquitinating and deubiquitinating enzymes (DUBs). DUBs are carefully regulated intra-cellular peptidases that cleave ubiquitin from target substrates. There are approximately 90 DUBs in the human genome, in 5 different families (Clague et al., 2013; Komander et al., 2009). The most abundant are the ubiquitin specific proteases (USPs) with 60 members that share a conserved USP catalytic domain, in which the ubiquitin core is held by the "fingers" while the catalytic centre lies between the "palm" and "thumb" subdomains. Despite this common catalytic core, the USP family members have many different modes of regulation (Sahtoe and Sixma, 2015).

Such regulation can take place in different ways and at different sites. USPs are regulated by changes in the catalytic domain, where the catalytic triad may be misarranged (Hu et al., 2002), via additional domains within the protein itself (Clerici et al., 2014; Faesen et al., 2011a), via post translational modifications (Nicassio et al., 2007), or by sub-cellular localization (Row et al., 2007). An interesting form of regulation is seen in a small subfamily of USPs, that includes USP1, USP12 and USP46. These three proteins are activated by complex formation with a WD40 repeat protein called UAF1 (USP1 associated factor, also known as WDR48) that leads to increased catalytic turnover for these enzymes (Cohn et al., 2007).

USP12 and USP46 are small proteins (370 and 366 residues respectively) with a highly conserved catalytic domain and high sequence similarity (88% identity). The related paralog USP1 (31% identity) is much larger (785 residues), due to additional inserts within its catalytic domain. The USP12/UAF1 and USP46/UAF1 complex can be further activated by binding to a second WD repeat protein, WDR20. This hyper-activation is not observed for the USP1/UAF1 complex (Kee et al., 2010). The lack of hyper-activation in USP1 could be due to the presence of the long inserts which might prevent it from interacting with WDR20. All three enzymes have low intrinsic activity in isolation and binding to UAF1 leads to activation which was shown to be due to an increase in k_{cat} with no drastic change in the K_M (Cohn et al., 2007; Faesen et al., 2011b; Villamil et al., 2012a). The exclusive k_{cat} activation is unusual for DUBs as most intermolecular activators (except GMPS) affect substrate binding (Faesen et al., 2011b).

The UAF1 regulation of this subfamily of DUBs has attracted considerable attention due to the biological importance of the enzymes involved. USP1 is important in DNA repair, where it acts

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http://dx.doi.org/10.1016/j.jsb.2016.09.011
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Please cite this article in press as: Dharadhar, S., et al. A conserved two-step binding for the UAF1 regulator to the USP12 deubiquitinating enzyme. J. Struct. Biol. (2016), http://dx.doi.org/10.1016/j.jsb.2016.09.011

on mono-ubiquitinated FANCD2 and PCNA in DNA cross-link repair and DNA-damage avoidance pathways (Huang et al., 2006; Nijman et al., 2005). It is also found to deubiquitinate the ID family of transcriptional regulators (Williams et al., 2011). Due to these important functions, USP1 is considered a major possible drug target (Liang et al., 2014). USP46 plays important roles in neurobiology, as a small deletion mutation in USP46 leads to neurological effects in mice, including anxiety and changes in learning and memory (Imai et al., 2013; Zhang et al., 2011). The molecular basis for these effects is not yet clear. For USP12 several possible roles have been described. It is involved in stabilizing the Akt phosphatases resulting in decreased levels of pAkt (Gangula and Maddika, 2013). It has also been reported that USP12 and USP46 deubiquitinate histone H2A and H2B thereby playing a role in Xenopus development (Joo et al., 2011). Recently USP12 was shown to stabilize the T-cell receptor complex at the cell surface by deubiquitinating TCR adaptor proteins LAT and Trat1 (Jahan et al., 2016).

Several studies have tried to uncover the detailed mechanism of USP1/12/46 activation by UAF1 and also the interfaces involved in the formation of this complex. It was suggested that UAF1 binding modulates the active site conformation of USP1 resulting in a productive catalytic triad and also that phosphorylation of Ser313 is necessary for its interaction (Villamil et al., 2012a,b). Other studies have shown that the regions in and around the fingers domain of USP1 might be necessary for UAF1 binding (Olazabal-Herrero et al., 2015). Recently the crystal structure of the USP46-Ub and its complex with UAF1 were determined. In these structures two possible interfaces for UAF1 binding and activation were identified. The authors used mutational analysis to show that Interface 1 is critical for UAF1 binding and activation (Yin et al., 2015).

Here we present the structure of USP12-Ub/UAF1$_{580}$ complex which was solved at a resolution of 2.8 Å and compare it to the UAF1 structure alone. Intriguingly, we find that these structures resemble the USP46/UAF1 complex structure (Yin et al., 2015), including the presence of the second binding site. We then show that in solution USP12 can bind to a second molecule UAF1, but with lower affinity. We confirm for USP12 that Interface 1 at the fingers site is the high affinity interface while the second low affinity interface could be at the backside of the ubiquitin binding cleft. Moreover, mutagenesis studies suggest that the first binding event at the fingers is responsible for activation while the second binding does not influence activity.

2. Materials and methods

2.1. Plasmids and cloning

Human USP12, USP46, USP1 and UAF1 constructs were obtained from Martin Cohn. The USPs were cloned into pFastbac-HTb vector and UAF1 was cloned into pFastbac1 (N-terminal Strep tag) for insect cell expression. The sequence verified insert containing pFastbac vectors were transformed into DH10Bac cells for bacmid preparation. The recombinant bacmid was used for transfection of *Spodoptera frugiperda* (*sf*9) insect cells to produce the recombinant baculovirus. USP12$_{WT}$ (24–370) and USP46 (8–366) were also cloned into the pGEX and pET bacterial expression vectors of the NKI LIC suite, respectively (Luna-Vargas et al., 2011). All USP12 mutants were made using the QuikChange site directed mutagenesis method on pGEX USP12 bacterial construct.

2.2. Protein expression and purification

N-terminal His-tagged USP1 (21–785) and USP12$_{FL}$ were expressed using Baculovirus expression in *sf*9 insect cells. After 72 h of infection the cells were harvested in 20 mM Tris (pH 8.0) + 200 mM NaCl + 2 mM TCEP + protease inhibitor (lysis buffer) and lysed by sonication. The lysed cells were spun down (21,000 rpm for 1 h) and the lysate was loaded on a His-affinity column (GE, USA). The column was washed with lysis buffer supplemented with 50 mM Imidazole (pH 8.0) followed by elution with lysis buffer supplemented with 500 mM imidazole. The His-USP1 elution fractions were concentrated and loaded on a size exclusion chromatography column (Superdex 200, GE, USA) equilibrated in GF buffer (20 mM HEPES (pH 7.5) + 150 mM NaCl + 2 mM DTT) following which the protein fractions were concentrated up to 4.5 mg/ml and stored in −80 °C. The USP12$_{FL}$ and USP46$_{FL}$ fractions were incubated with TEV protease and dialyzed overnight in lysis buffer without imidazole. The dialysed protein sample was then loaded on a His-affinity column where the protein was obtained in the wash fractions. The protein containing fractions were then concentrated and loaded on a size exclusion chromatography column (Superdex 200, GE, USA) equilibrated in GF buffer following which protein fractions were concentrated up to 6 mg/ml and stored in −80 °C.

N-terminal GST-tagged USP12$_{WT}$ and mutants were expressed in *E. coli*. The cells were induced with 0.2 mM IPTG at 18 °C for 10–12 h followed by which they were harvested in lysis buffer and lysed by sonication. The lysate was loaded on a GST-affinity column and washed with lysis buffer followed by elution with lysis buffer supplemented with 20 mM Glutathione. The protein containing fractions were then concentrated and loaded on a size exclusion chromatography column (Superdex 200, GE, USA) equilibrated in GF buffer following which protein fractions were concentrated up to 15 mg/ml and stored in −80 °C.

N-terminal His-tagged USP46 (8–366) was expressed in *E. coli*. The cells were induced with 0.5 mM IPTG at 20 °C for ~16 h followed by which they were harvested in 20 mM Tris (pH 7.5), 150 mM NaCl, 10 mM Imidazole (pH 8.0) and 5 mM beta-mercaptoethanol (lysis buffer) and lysed by sonication. The lysate was loaded on a Ni-affinity column and washed with lysis buffer supplemented with 50 mM imidazole followed by elution with lysis buffer supplemented with 500 mM imidazole. USP46 was incubated with TEV protease and dialysed overnight in lysis buffer without imidazole. The dialysed protein sample was then loaded on a His-affinity column where the protein was obtained in the wash fractions. USP46 was loaded on a size exclusion chromatography column (Superdex 200, GE, USA) equilibrated with 20 mM Tris (pH 7.5), 150 mM NaCl and 2 mM DTT.

UAF1$_{FL}$ and UAF1$_{580}$ were also purified from *sf*9 insect cells and were lysed in similar conditions as the USP purifications. The UAF1 lysates were loaded on a Strep-affinity column (IBA life sciences) and washed with 50 mM Tris (pH 8.0) + 150 mM NaCl + 2 mM TCEP (wash buffer). The strep-UAF1 protein was then eluted using wash buffer supplemented with 2.5 mM Desthiobiotin. The protein containing fractions were concentrated and loaded on a size exclusion chromatography column (Superdex 200, GE, USA) equilibrated in GF buffer. The fractions corresponding to the peak were collected. UAF1 samples were treated with 10 mM iodoacetamide to prevent background DUB activity due to minute amounts of co-purified insect cell DUBs. Following iodoacetamide treatment the protein fractions were re-purified using size exclusion chromatography (Superdex 200, GE, USA). The protein fractions were then concentrated up to 10 mg/ml and stored in −80 °C.

The USP/UAF1 complexes were co-expressed in baculovirus infected *sf*9 insect cells. USP/UAF1 complexes were purified by first carrying out a His-affinity purification followed by Strep-tag (IBA life Sciences) affinity purification as described above. The protein fractions were then concentrated and loaded on a size exclusion chromatography (Superdex S200, GE, USA) equilibrated in GF buffer. The protein containing fractions were concentrated up to 5 mg/ml and stored at -80 °C.

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