Ubiquitin-specific protease 4 is inhibited by its ubiquitin-like domain

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INTRODUCTION

Post-translational modification by the small, highly conserved ubiquitin (Ub) protein has an essential role in the regulation of many cellular processes in eukaryotes (Pickart, 2004; Hochstrasser, 2009). In this process, the carboxy-terminus of Ub forms an isopeptide with lysines on the target proteins, or on Ub itself, to form poly-Ub chains. The activity of the conjugating enzymes E1–E2–E3 is actively balanced through hydrolysis by deubiquitinating enzymes (DUBs; Amerik & Hochstrasser, 2004; Pickart, 2004; Nijman et al., 2005; Deshaies & Joazeiro, 2009; Komander et al., 2009). Deregulation of the ubiquitination pathway can lead to cancer and neurodegenerative diseases (Hoeller & Dikic, 2009; Avvakumov et al., 2006; Renatus et al., 2006; Kohler et al., 2010; Samara et al., 2010).

USPs are variable in size with modular domain architecture including, for example, TRAF-like, DUSP or Znf domains (Nijman et al., 2005; Komander et al., 2009). Sequence analysis predicted the presence of Ub-like (Ubl) domains in 17 different USPs (Zhu et al., 2007). Integrated Ubl domains are stretches of 45–80 amino acids that share the β-grasp fold of Ub, but often have poor sequence conservation among subfamilies (Kiel & Serrano, 2006; Burroughs et al., 2007). The Ubl domains in the USP family are located amino-terminally, within or C-terminally to the catalytic domain. Structural studies of the N-terminal Ubl domain of USP14 confirmed the Ubl-fold (Protein Data Bank (PDB): 1WGG) and showed involvement in proteasome binding that promotes the DUB activity of USP14 (Hu et al., 2005). Similar to USP14, USP4 has a Ubl domain N-terminal of its catalytic domain, but it has an additional Ubl domain embedded in the catalytic domain.

USP4 was previously known as ubiquitous nuclear protein (UNP) (Gupta et al., 1993). Identified as a proto-oncogene related to Tre2/Tre17 (USP6), USP4 shows a consistently elevated gene expression level in small cell tumours and lung adenocarcinomas, suggesting that it may have a possible causative role in neoplasia (Gray et al., 1995). Besides possible roles in Wnt signalling (Zhao et al., 2009) and recruitment to the A2A receptor (Milojevic et al., 2006), USP4 is recruited to the spliceosome by complex formation with Sart3 (Song et al., 2010). Here, it preferentially deubiquitimates K63-linked chains on the U4 component Prp3. Another component of the spliceosome complex is the catalytically inactive USP39 (Sowa et al., 2009; Song et al., 2010), which controls the messenger RNA levels of Aurora B (van Leuken et al., 2008).

Here, we report on the crystal structure of the catalytic domain of USP4 without the internal Ubl domain, and show how this Ubl domain is likely to inhibit the catalytic activity of USP4.
domain acts as an autoregulatory domain that partially inhibits catalytic activity by competitive inhibition.

RESULTS
Identification of USP4–D1D2
To gain insight into the structure and function of USP4, we expressed and purified the USP4 catalytic domain (amino acids 296–954, Fig 1A) in Escherichia coli. To improve the chances for crystallization, we used limited proteolysis. After treatment with thermolysin, two fragments—domain 1 (D1) and 2 (D2)—were obtained, which copurified on size exclusion chromatography and together retained DUB activity (supplementary Fig S1A,B online). We identified the composition of D1 and D2 using mass spectrometry and N-terminal sequencing (supplementary Fig S1C online) and compared them against a multi-sequence alignment of USP family members. This showed that the protease treatment removed an insertion between Leu 481 and Leu 766 (supplementary Fig S2 online), yielding a minimal catalytic domain consisting of an enzymatically active complex of two fragments: USP4–D1D2.
Structure of the USP4–D1D2 catalytic domain

We crystallized and determined the USP4–D1D2 structure by molecular replacement using the USP8 catalytic domain (PDB: 2GF0) as the search molecule, and refined it to 2.4 Å resolution with an R/Refrac of 0.178/0.21 and good geometry (Fig 1B; supplementary Table S1 online). There are six molecules of USP4–D1D2 per asymmetrical unit, with a pairwise root-mean-square deviation of approximately 0.7 Å over 344 residues using the PISA program (Krissinel & Henrick, 2004).

Similar to crystal structures of other USPs, the catalytic domain of USP4–D1D2 resembles an extended right hand comprising three domains: Fingers, Thumb and Palm (Fig 1B; supplementary Fig S3 online). The D1 fragment contains the Thumb domain and part of the Fingers domain with the Cys box (amino acids 303–320) and QEQD box (amino acids 390–403) of the active site, whereas the D2 fragment completes the active site with the His box (amino acids 864–885, 894–903, 915–922) and makes the remaining part of the Fingers and the Palm (Quesada et al, 2004; supplementary Fig S2 online). Like other USP structures (Hu et al, 2005; Avvakumov et al, 2006; Renatus et al, 2006; Köhler et al, 2010; Samara et al, 2010), except USP7 (Hu et al, 2002), the catalytic triad is in a catalytically competent configuration, wherein His711-ND1 is 3.2 Å away from Cys311-SG and His711-ND2 is hydrogen bonding with Asp898-OD1 (2.7 Å; Fig 1B).

The zinc-finger ribbon observed in USP2 and USP8 is present in USP4 (Fig 1B,C). The Zn²⁺ ion brings together the D1 and D2 domains, tetrahedrally coordinated by cysteines on anti-parallel β-strands β1 and β2 in D1, and β4 in D2. This zinc-finger ribbon in the Fingers domain seems to be in the contracted ‘closed-hand’ configuration seen in USP8 that blocks access of Ub to its binding site (Avvakumov et al, 2006). A similar role was assigned to the two Ub-binding surface loops (BL1 and BL2) in USP14 (Hu et al, 2002) that block the active site, but relocate after Ub binding. In USP4, both loops (Fig 1D)—as well as a third blocking loop (BL3) that hinders access of the C-terminal tail of Ub to the binding pocket—are observed.

Superposition of the six non-crystallographic symmetry-related molecules of USP4–D1D2 shows that both the zinc-finger ribbon and the three blocking loops show flexibility (maximal Cα displacement 4 Å; Fig 1C,D), which is in agreement with their role in activation (Hu et al, 2006; Avvakumov et al, 2006).

The insert inhibits deubiquitinating activity

We compared the catalytic activity of the USP4 catalytic domain with and without the large insert, by using in vitro deubiquitinating assays. In these assays we followed the hydrolysis of K63- and K48-linked di-Ub in mono-Ub (Fig 2A,B; supplementary Fig S4A,B online). We observed that K63 di-Ub is more efficiently degraded than K48, in agreement with the role of USP4 in splicing (Song et al, 2010). Interestingly, quantification (Fig 2D; supplementary Fig S4D online) shows that USP4–D1D2 without insert is more efficient at degrading both di-Ubs than the complete catalytic domain. When D1 and D2 are fused through a short linker, as found in USP7 (supplementary Fig S2 online), their activity is similar to that of USP4–D1D2, showing that the cause of the activation is the lack of insert and not the chain break (Fig 2C; supplementary Fig S4C online).

In Ub-7-amido-4-methylcoumarin (Ub-AMC) assays the intact USP4 catalytic domain is also less active than USP4–D1D2 or the fusion protein. As only AMC is cleaved off, the inhibition is not dependent on the protein target. When analysed by Michaelis–Menten kinetic analysis (Fig 2E), the Vmax values were similar, but the Km for the intact catalytic domain (13.5 μM) was weaker than that for USP4–D1D2 (0.20 μM), leading to approximately 90-fold lower catalytic efficiency overall (kcat/Km) for USP4CD than for USP4–D1D2.

As the insert seems to inhibit the DUB activity of USP4, we tested whether it could do so in trans. We expressed and purified the insert (amino acids 483–765) and added it in increasing amounts to USP4–D1D2 in the Ub-AMC assay (supplementary Fig S5A online). We observed that the insert slows deubiquitination by USP4–D1D2. To investigate whether this reduction in DUB activity is due to molecular crowding, we repeated the in trans inhibition assay with USP4–D1D2 in the presence of either SUMO or BSA (supplementary Fig S6 online). Neither of these reduced DUB activity, confirming that the insert is intrinsically able to inhibit the catalytic activity of USP4.

Competitive inhibition of the USP4 insert

We tested whether USP4–D1D2 would directly interact with the insert. In a surface plasmon resonance (SPR; Fig 3B) experiment, we observed binding of USP4–D1D2 to the insert, with a Keq of 1.32 μM after equilibrium fitting. This affinity closely resembled the affinity of USP4–D1D2 for Ub itself (Keq of 1.39 μM; Fig 3A).

Therefore, we tested whether the insert could compete with Ub for binding to USP4–D1D2, and would therefore bind to the same binding site. In an SPR competition experiment we flowed USP4–D1D2 over a glutathione S-transferase (GST)-tagged insert in the presence of increasing amounts of Ub (Fig 3D). We observed decreasing binding of USP4–D1D2 to the GST-insert as the Ub concentration increased. The data could be fitted with a one-site competition binding model with a Keq of 1.4 μM, showing that the USP4 insert competes with Ub for binding to USP4–D1D2.

Interestingly, the Keq of intact USP4CD for Ub is only fourfold less, compared to USP4–D1D2 in an isothermal titration calorimetry (ITC) experiment (Fig 3E). Although the exact Keq are slightly tighter in the ITC experiment, qualitative analysis of SPR experiments agrees with this assessment. Non-specific binding at high concentrations precluded detailed fitting of these data (supplementary Fig S7 online), but the curves show that binding of Ub to USP4CD has a slower off-rate than that of Ub to USP4–D1D2, and together with the Keq value also suggest that it has a slower on-rate. As the Km is dependent on Keq as well as the binding rate, the combination of slow kinetics and slightly lower affinity explains the differences in Keq values. Apparently, the insert prevents rapid binding as well as rapid release of the Ub substrate, allowing competitive binding.

Finally we analysed whether the enzymatic activity is competitively inhibited by the addition of the insert in trans. We tested the enzymatic activity with varying inhibitor concentrations against a range of substrate concentrations (Fig 2F), and fitted the data against different inhibition models (Copeland, 2000). We tested the enzymatic activity with varying inhibitor concentrations against a range of substrate concentrations (Fig 2F), and fitted the data against different inhibition models (Copeland, 2000). We tested the enzymatic activity with varying inhibitor concentrations against a range of substrate concentrations (Fig 2F), and fitted the data against different inhibition models (Copeland, 2000). We tested the enzymatic activity with varying inhibitor concentrations against a range of substrate concentrations (Fig 2F), and fitted the data against different inhibition models (Copeland, 2000).
Ub2–K63 disassembly

10

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D2

0

× 5.9 (D2 Ubl D2 et al in vivo in trans 1 0 0.68)

× 10

in trans between USP4CD and D1D2, we observed a 90 times lower enzyme efficiency for the insert containing USP4CD. (Insert inhibits the DUB activity of USP4CD. (K in Ub-AMC assays at different Ubl-insert concentrations (5, 15, 45 and 90 units)

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additional conformational changes take place. One possibility is that the enzyme reaches a state after turnover that has lower affinity for the insert, and is therefore not as effectively inhibited.

The Ubl domain is sufficient for inhibition

The presence of a Ubl domain within the insert was predicted (supplementary Fig S2 online; Zhu et al, 2007). To test whether the Ubl domain is sufficient for binding to the USP4 catalytic domain, we performed the SPR experiment with the purified Ubl domain (amino acids 483–571, Fig 1A) and found a K_d of 1.36 µM towards USP4–D1D2, which is similar to that for the complete insert (Fig 3C). This suggests that the Ubl domain is the functional part of the insert.

To test whether the Ubl domain can inhibit the DUB activity of USP4, we repeated the in trans inhibition assay with USP4–D1D2 in the presence of increasing amounts of the Ubl domain (supplementary Fig S6 online) and found that it provides inhibition equal to the insert. We therefore conclude that the Ubl domain is sufficient to inhibit the DUB activity of USP4, through competitive inhibition of Ub binding.

Regulation by other USP enzymes

As the Ubl domain seems to bind in the substrate Ub-binding site of USP4, we wondered whether other USP enzymes could also bind to the Ubl domain. We tested whether our Ubl domain containing insert could bind to the catalytic domain of USP39 and USP8, and found similarly high affinities as for USP4CD (Fig 4B,C).

Then, we analysed whether these DUBs could modulate USP4CD activity. We repeated the in trans Ub-AMC assay with USP4CD in the presence of the intrinsically inactive USP39CD or an inactive variant of the USP8 catalytic domain, USP8CD-mut (Fig 4A). For both USPs we observe a modest activation of USP4CD that was dependent on the presence of the Ubl-containing insert, as it does not increase the DUB activity of USP4–D1D2 in this manner.

Apparently, other USP enzymes can regulate USP4 activity by competing for binding to the Ubl domain. This effect could be larger when the USPs have further interactions. As USP39 forms a stable complex with USP4 in cells (Sowa et al, 2009; Song et al, 2010), it is a prime candidate for an activating role in vivo.
**DISCUSSION**

We show that the predicted Ubl domain within a large insert embedded in the USP4 catalytic domain partially inhibits DUB activity by competing with Ub for binding. Superposition of the crystal structure of USP4-Δ1D2 and any Ubl domain on USP7 in complex with Ub-aldehyde (PDB: 1NBF), respectively, shows that the Ubl domain would fit like a Ub molecule into the hand of USP4-Δ1D2 (Fig 5A), only requiring movements in the blocking loops and the zinc-finger ribbon. Hence, we propose a model in which the Ubl domain partially inhibits DUB activity through competitive inhibition by binding into the hand of USP4 and thus preventing Ub substrate binding (Fig 5B).

This function of an integrated Ubl domain is relatively new. The Ubl domains in proteasomal shuttle factors Rad23 and Dsk2, as well as in Parkin and USP14, function in recruitment of ubiquitinated proteins to the proteasome (Sakata et al., 2003; Hu et al., 2005). Other Ubl domains regulate the enzymatic activities of immune-response inducible kinases such as IKKβ (a subunit of IkB kinase complex; May et al., 2004), or as PB1 (Phox and Bem1) domains, have a role in the regulation of
signal transduction in proteins such as P62, MEK5 and protein kinase C (Terasawa et al., 2001; Sumimoto et al., 2007). However, all these Ubl-domain families have low sequence similarities, indicating that their functions are probably distinct between subfamilies.

The activity of USPs is regulated through an inactive conformation of the catalytic triad, as in USP7, or through a series of blocking loops or a blocking zinc-finger ribbon. USP4 seems to combine the blocking loops and zinc-finger ribbon with a further regulation through the Ubl domain.
Whether Ubl domains provide a common regulation mechanism for the DUB activity of USPs is an interesting question for future research. A second Ubl domain is found within USP4, at its N-terminus. A recent crystal structure (PDB: 3JYU, amino acids 139–226) shows that this Ubl domain interacts extensively with the adjacent DUSP (domain in USP) domain (amino acids 27–125). This region of the protein is primarily important for interaction with Sar3 (Song et al, 2010) and hence might not have this function.

However, USP4 is not the only DUB with a Ubl fold within its catalytic domain. Sequence analysis by Zhu et al (2007) identified an integrated Ubl fold within the catalytic domain of USPs 6, 11, 15, 19, 31, 32 and 43, embedded in a larger insert, like in USP4. In particular, USP11 and USP15 are closely related to USP4. This subgroup of USPs probably also regulates DUB activity through its Ubl domain.

The way in which Ubl-domain inhibition itself is regulated is an exciting question. One could imagine that further post-translational modification by, for example, phosphorylation or acetylation would enable the release of the full activity of the DUB enzyme. In addition, we have shown that binding partners such as USP39, can activate USP4 function by binding to the Ubl domain (Fig 5C). Although the activation is modest (Fig 4A), this could be increased by further interactions, as observed in the spliceosome complex.

Regardless of the mechanisms that regulate USP4 activation, it is clear that this is true of internal regulation by a Ubl domain allows the creation of an extremely fast response element to external signals.

METHODS

For plasmids, cloning, protein preparation and identification, crystallization and structure determination procedures, see supplementary information online and references.

**Ub-AMC assays.** Quantitative activity (triplicate) and inhibition (duplicate) assays or USP modulation assays (triplicate) were performed using Ub-AMC with 10 nM enzyme in 30 µl reaction volume in 384-well plates and preincubated for 15 min at 21 °C, for inhibition assays with UBl insert and with other DUBs for modulation assays. Initial velocities against Ub-AMC concentration were computed to derive steady-state kinetic parameters using GraphPad Prism5 (GraphPad Software Inc.). Non-linear fit of four inhibition models was compared in GraphPad.

**Di-Ub assays.** K48 and K63 di-Ub substrates were produced and purified as described (Rassi & Pickart, 2005). 75 nM enzyme was incubated with 3 µM di-Ub, subjected to SDS-polyacrylamide gel electrophoresis and image analysis, and quantitation was performed in duplicate with TINA 2.09 (Raytest Co.).

**SPR and ITC.** SPR was performed on a Biacore T-100, with GST-Ub, GST-insert and GST-Ubl domain immobilized on anti-GST antibodies coupled to a CM5 chip. Data (duplicate) were processed using BioEvaluation (GE Healthcare) and GraphPad Prism5. ITC was performed on a VP-ITC Micro Calorimeter (MicroCal, Inc.) by titrating Ub into USP4-D1D2 or USP4CD.

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**Supplementary information** is available at EMBO reports online (http://www.emboreports.org).

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**CONFLICT OF INTEREST**

The authors declare that they have no conflict of interest.

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