Ubiquitin binding proteins protect ubiquitin conjugates from disassembly

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Abstract As a step in their turnover proteins in eukaryotic cells are coupled to a small protein, ubiquitin, before they are recognised by 26S proteasomes and degraded. However, cells also contain many deubiquitinating enzymes, which can rescue proteins by cleaving off the ubiquitin chains. Here we report that three ubiquitin binding proteins, Rhp23, Dph1 and Pus1, from fission yeast can protect multiubiquitin conjugates against deubiquitination. This protection depends on the ubiquitin binding domains and may promote degradation of ubiquitinated proteins.

Key words: Ubiquitin; Proteasome; Deubiquitinating enzyme; Ubiquitin-C-terminal hydrolase; Ubiquitin pathway associated domain; Ubiquitin interaction motif

1. Introduction

Protein degradation in eukaryotic cells is important for many regulatory mechanisms [1]. Before they are degraded, proteins are marked for destruction by being furnished with a chain of ubiquitin. The multiubiquitin gives them affinity for the 26S proteasome, a multisubunit protease responsible for degradation of most cell proteins [1].

Ubiquitin ligation is accomplished in multiple steps. Initially the E1 enzyme activates ubiquitin in an ATP-consuming process. Ubiquitin is then transferred to an E2 enzyme and finally to the target protein in a process catalysed by an E3 enzyme [2]. Several rounds of conjugation yield substrates carrying chains of ubiquitin moieties. A range of different E2s and E3s confer substrate specificity to the process.

The 26S proteasome consists of a proteolytically active 20S cylinder with 19S regulatory ATPase complexes attached to the ends [3,4]. The 19S complexes regulate the access to the proteolytic cylinder [4] and recognise substrates [5,6] which usually must be conjugated to a chain of at least four ubiquitin moieties [7].

Several deubiquitinating enzymes appear to play important regulatory roles in trimming the length of ubiquitin chains on target proteins [8]. Ubiquitination therefore reflects the balance between multiubiquitination and either degradation of conjugates by the 26S proteasome or disassembly of the ubiquitin chains by deubiquitinating enzymes. Hence, deubiquitination of a substrate may render it unrecognisable to the 26S proteasome and rescue it from degradation.

A number of ubiquitin binding proteins have been described [9,10] and the protein domains involved in ubiquitin binding have been characterised [11–13]. One of these proteins is the subunit of the 26S proteasome called S5a in mammals [9] and Rpn10 or Pus1 in budding and fission yeast, respectively [14,15]. It binds ubiquitin chains via a ubiquitin interaction motif (UIM) [11] and appears to work as a substrate binding site for the 26S proteasome [5,9,10]. In yeast [14,15], but not in mammals [16], S5a/Rpn10/Pus1 is found as a free protein, besides being incorporated into the 26S proteasome.

The proteasome interacting proteins called Rad23 and Dsk2 in budding yeast and Rhp23 and Dph1 in fission yeast also bind multiubiquitin. Rad23/Rhp23 and Dsk2/Dph1 utilise a ubiquitin pathway-associated (UBA) domain for ubiquitin binding [10]. Like the UIM domain, the UBA domain preferably binds chains composed of at least four ubiquitin moieties and both Rad23/Rhp23 and Dsk2/Dph1 appear to target substrates to the 26S protease [10,17–19].

Here we report that in the fission yeast *Schizosaccharomyces pombe*, Rhp23 and Dph1 protect ubiquitin chains against disassembly by deubiquitinating enzymes. Pus1 also protects against deubiquitination, but much less efficiently. This inhibition of deubiquitination depends on the ubiquitin binding domains and is the first example of naturally occurring protectors of multiubiquitin chains.

2. Materials and methods

2.1. Protein expression and purification

The expression plasmids were: ubiquitin-specific protease Y (UBPY) [20], Rhp23, Rhp23UBA1, Rhp23UBA2, Rhp23PP, Dph1, Pus1, Pus1UIM and Pus1N5 [10,15], all subcloned into the pGEX-KG vector (Amersham-Pharmacia).

Glutathione *S*-transferase (GST) fusion proteins were expressed in *Escherichia coli* BL21 (DE3) pLysS, bound to glutathione Sepharose 4 beads (Amersham-Pharmacia) and released with thrombin (Amersham-Pharmacia) as described by the manufacturer. Protein concentrations were determined [21] with bovine serum albumin as a standard.

2.2. UBPY binding experiments

Approximately 10 μg of GST or GST-tagged UBPY on glutathione Sepharose 4 beads was incubated overnight at 4°C in buffer A (50 mM Tris–HCl pH 7.5, 100 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 2 mM dithiothreitol (DTT) and 1 mM phenylmethylsulphonyl fluoride (PMSF)) with 1 μg of purified Rhp23, Dph1 or

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Abbreviations: AMC, 7-amino-4-methylcoumarin; DUB, deubiquitinating enzyme; GST, glutathione *S*-transferase; Ubal, ubiquitin aldehyde; UBA, ubiquitin pathway-associated domain; UBP, ubiquitin-specific protease; UIM, ubiquitin interaction motif

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Pus1. The beads were washed in buffer A, bound proteins separated on 12.5% sodium dodecyl sulphate (SDS) gels and analysed by Western blotting with rabbit antisera against Rhp23, Dph1 and Pus1 (not shown).

2.3. Deubiquitination assays

Aliquots of 1 μg K48-conjugated tetraubiquitin chains (Affiniti Research Products) were incubated overnight at 37°C with 1 μg of UBPY in 30 μl buffer B (50 mM Tris–HCl pH 8.3, 5 mM $MgCl_2$, 2 mM DTT and 2 mM NaN_3) containing ubiquitin binding proteins or ubiquitin aldehyde (Ubal) (Affiniti Research Products) at a concentration of 5 μM.

Degradation products were analysed by SDS–polyacrylamide gel electrophoresis (PAGE) and Western blotting with rabbit anti-ubiquitin antibody. A mixture of mono-, di-, tri-, tetra-, penta-, hexa- and heptaubiquitin (Ub_{1-7}) (Affiniti Research Products) was used as a marker.

For ubiquitin-7-amino-4-methylcoumarin (ubiquitin-AMC) (Affiniti Research Products) deubiquitination assays, samples were incubated for 30 min at 37°C in 200 μl of 0.5 μM ubiquitin-AMC, 50 mM Tris–HCl pH 8.0, 5 mM DTT and 0.1% Tween-20. After addition of 100 μl 10% SDS and 1.5 ml 0.1 M sodium borate pH 9.1, the fluorescence was determined at an excitation wavelength of 380 nm and an emission of 460 nm. Less than 5% of the substrate was cleaved during the incubation (not shown).

For deubiquitination assays in cell extracts the temperature sensitive *S. pombe* proteasome mutant *mts3-1* [22] strain was grown at 28°C to mid-exponential phase in YPD. The cells were then incubated for another 24 h at 35°C and lysed using glass beads in ice cold buffer C (50 mM Tris–HCl pH 7.5, 100 mM NaCl, 10% glycerol, 2 mM DTT, 1 mM PMSF, EDTA-free Complete™ protease inhibitors (Roche) and 50 μM of the proteasome inhibitor Z-Ile-Glu(OtBu)-Ala-Leu-aldehyde (Bachem)). To 50 μl cell extract (1 mg protein/ml) 2 μg of either Rhp23, Dph1 or Pus1 in buffer C was added. After 3 h at 37°C, proteins were separated by SDS–PAGE and analysed by Western blotting with rabbit anti-ubiquitin antibody.

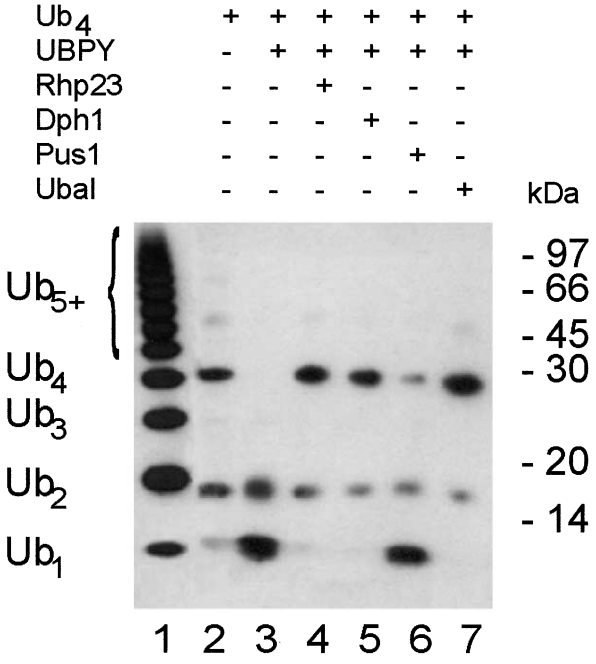

Fig. 1. Protection of tetraubiquitin chains. Deubiquitination of tetraubiquitin by UBPY without addition (lane 3) or in the presence of the ubiquitin binding proteins Rhp23 (lane 4), Dph1 (lane 5), Pus1 (lane 6) or the inhibitor Ubal (lane 7). The degradation products were analysed by SDS–PAGE and Western blotting with an anti-ubiquitin antibody. Tetraubiquitin chains without UBPY were not disassembled (lane 2). For comparison a mixture of mono- (Ub), di- (Ub_2), tri- (Ub_3), tetra- (Ub_4), penta- (Ub_5), hexa- (Ub_6) and heptaubiquitin (Ub_7) was loaded (lane 1).

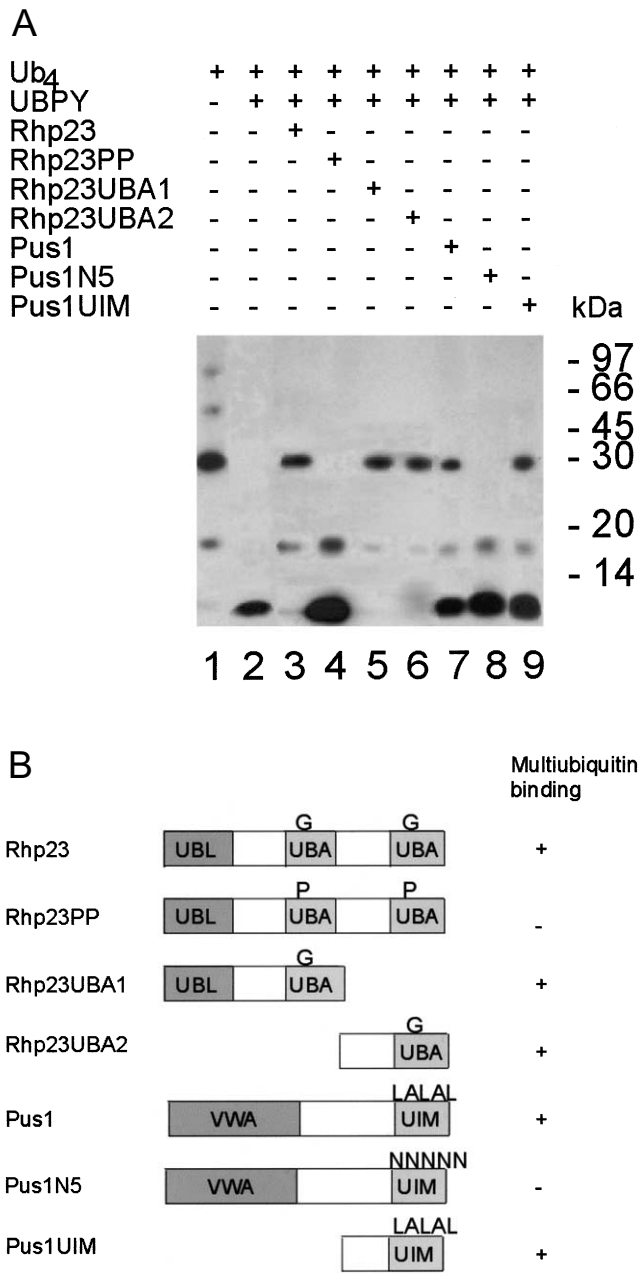

Fig. 2. Inhibition is dependent on ubiquitin binding. A: Deubiquitination by UBPY of tetraubiquitin chains (lane 2) was performed in the presence of the ubiquitin binding proteins Rhp23 (lane 3) or Pus1 (lane 7). No inhibition of deubiquitination is seen with Rhp23PP (lane 4) or Pus1N5 (lane 8) which carry point mutations abrogating ubiquitin binding in the UBA and UIM domains respectively. Alone the two UBA domains of Rhp23 (Rhp23UBA1 and Rhp23UBA2) (lanes 5 and 6) or the UIM domain of Pus1 (Pus1-UIM) (lane 9) inhibit deubiquitination as efficiently as the full length proteins (lanes 3 and 7). Tetraubiquitin chains without UBPY were not disassembled (lane 1). B: The domain organisation of the various expression constructs is shown in a schematised form (not to scale). These proteins have previously been assayed for binding to multiubiquitin chains [10,15].

3. Results

Overexpression of Rhp23 and Dph1 causes a dramatic increase in the level of ubiquitinated proteins in vivo [17,23–26]. This effect might be due to an inhibition of either the 26S

proteasome or the deubiquitinating enzymes (DUBs) or both.

To investigate the effect of ubiquitin binding proteins on deubiquitination in vitro we set up a deubiquitination assay using human UBPY [20] and tetraubiquitin chains conjugated through lysine 48. UBPY disassembled tetraubiquitin chains (Fig. 1, lanes 2 and 3) and was efficiently inhibited by the specific DUB inhibitor Ubal (Fig. 1, lane 7). However, when added in equimolar amounts with the substrate, Rhp23, Dph1 and less so Pus1 inhibited deubiquitination (Fig. 1, lanes 4–6).

Next we wished to determine whether the inhibitory effects of Rhp23, Dph1 and Pus1 on deubiquitination depend on their multiubiquitin binding. Certain point mutants in the ubiquitin binding sites of Rhp23 and Pus1 are unable to interact with ubiquitin chains [10,15]. Using these mutant proteins we now show that ubiquitin binding is essential for the proteins' ability to inhibit deubiquitination (Fig. 2). In the Rhp23PP mutant, where a conserved glycine residue in each of the UBA domains has been mutated to proline [10], the inhibition of UBPY-mediated chain disassembly was abrogated (Fig. 2A, lane 4). Similarly, when we used a Pus1N5 mutant, where the conserved LALAL motif in the UIM domain has been changed to five consecutive asparagine residues [15], the inhibitory effect was also no longer visible (Fig. 2A, lane 8).

Moreover, using truncated versions of Rhp23 and Pus1 which encode the ubiquitin binding domains alone (Rhp23UBA1, Rhp23UBA2 and Pus1UIM respectively, Fig. 2B), we could show that the UBA domains of Rhp23 and the UIM domain of Pus1 were sufficient to protect the tetraubiquitin chains against deubiquitination by UBPY and were as efficient as the full length proteins (Fig. 2). Each of the two UBA domains of Rhp23 is able to bind multiubiquitin [10], and both appear about equally efficient in inhibiting deubiquitination (Fig. 2A, lanes 5 and 6).

The inhibitory proteins had no direct effect on the activity of the deubiquitinating enzyme: the UBPY enzyme cleaved the fluorogenic substrate ubiquitin-AMC and was inhibited by Ubal (Fig. 3). However, no significant effects were observed when Rhp23, Dph1 or Pus1 were added in equimolar amounts with the substrate (Fig. 3) or even at concentrations 50 times higher (not shown).

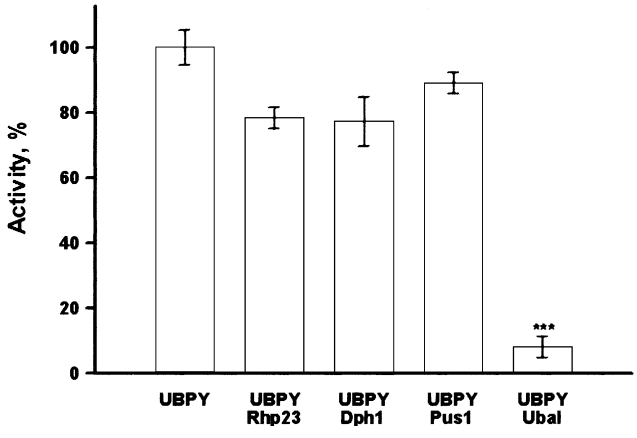

Fig. 3. Ubiquitin-AMC hydrolysis is not inhibited. Hydrolysis of the ubiquitin-AMC substrate by the ubiquitin-specific protease UBPY in the presence or absence of the ubiquitin binding proteins Rhp23, Dph1, Pus1 and the inhibitor Ubal. The data are shown as averages ± S.E.M., $n = 5$. In the presence of Ubal the hydrolysis was inhibited (***$P < 0.001$, Student's t-test), whereas the ubiquitin binding proteins had no significant effects.

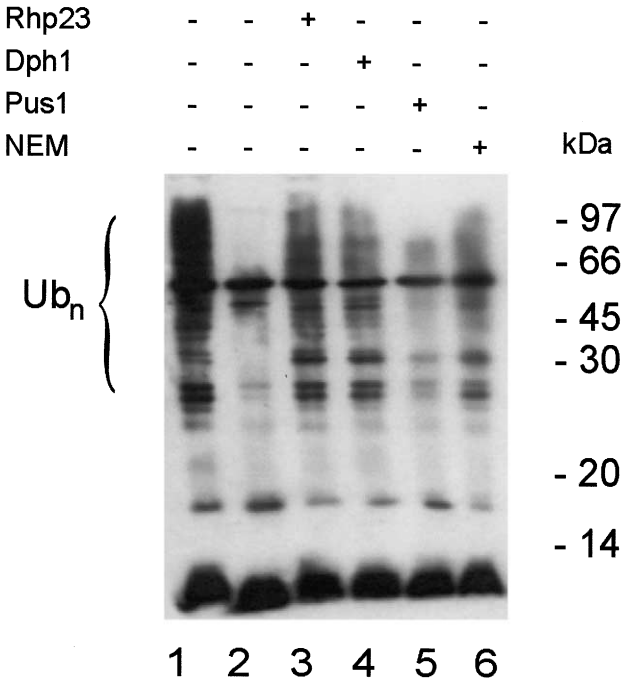

Fig. 4. Inhibition of ubiquitin chain disassembly in extracts. In the presence of proteasome inhibitor high molecular weight ubiquitin conjugates in the *mts3-1* mutant (lane 1) become disassembled by intrinsic ubiquitin-specific proteases during 3 h incubation at 37 °C (lane 2). Disassembly is retarded by Rhp23 (lane 3), Dph1 (lane 4) and less so by Pus1 (lane 5). In the presence of NEM deubiquitination is inhibited (lane 6).

Conceivably Rhp23, Dph1 or Pus1 might inhibit deubiquitination by binding UBPY. However, in precipitation experiments we could not detect any such interactions (not shown).

Next we sought to test whether our previous data could be reproduced in fission yeast extracts. Cell extracts from the proteasome mutant *mts3-1* [22] strain grown at the restrictive temperature contain high molecular weight ubiquitin–protein conjugates (Fig. 4, lane 1). The ubiquitin conjugates disappeared upon incubation (Fig. 4, lane 2) because of intrinsic deubiquitinating enzymes in the cell extract. The alternative explanation, that the conjugates were degraded by 26S proteasomes, can be ruled out because proteasomes from the *mts3-1* mutant are inert at the temperature used here [27] and the extract contained proteasome inhibitor as a further precaution. Confirming our previous results, addition of Rhp23 and Dph1 stabilised the ubiquitin conjugates (Fig. 4, lanes 3 and 4), as did *N*-ethylmaleimide (NEM), a potent inhibitor of most deubiquitinating enzymes (Fig. 4, lane 6). However, again the effect of Pus1 was not as strong (lane 5).

4. Discussion

In order to be degraded by the 26S proteasome proteins must carry ubiquitin chains composed of at least four ubiquitin moieties [7]. When the proteasome degrades the multiubiquitin-tagged substrate, the ubiquitin chains become detached. Several isopeptidases or ubiquitin-specific proteases

associated with 19S particle of the 26S proteasome presumably mediate this deubiquitination and protein degradation and multiubiquitin release seems to be tightly coupled [28–34].

The budding yeast genome encodes at least 17 different deubiquitinating enzymes and higher eukaryotes contain several more [35]. Some of the deubiquitinating enzymes trim ubiquitin chains sequentially from the distal end [28]. This ubiquitin chain editing process potentially regulates the substrate's association time with the 26S proteasome and could spare substrates from degradation if the ubiquitin chains become so short that they lose affinity for the proteasome (discussed in [36]).

Genetic and biochemical evidence shows that three ubiquitin binding proteins, Rhp23, Dph1 and Pus1, help target ubiquitinated proteins to the 26S proteasome [10]. These proteins all preferentially bind ubiquitin chains of four or more ubiquitin moieties, and their combined deletion results in the accumulation of multiubiquitin conjugates and is lethal in fission yeast [10]. Moreover, experiments have shown that when Rad23 and Dsk2, the Rhp23 and Dph1 orthologues in budding yeast, are overexpressed, some proteins are stabilised and multiubiquitin conjugates accumulate [17,23–26]. Since this effect is independent of whether the proteins interact with 26S proteasomes [17] it is hardly due to inhibition of proteasomes. The stabilisation of proteins may instead be caused by shifting of an equilibrium so that a larger proportion of the ubiquitin conjugates remain bound to Rhp23, Dph1 and Pus1 rather than being transferred to the proteasomes. The accumulation of multiubiquitin conjugates might also be caused by an inhibition of deubiquitination and our results did indeed show that Rhp23, Dph1 and Pus1 inhibit deubiquitination, both by the mammalian enzyme UBPY and by deubiquitinating enzymes in yeast extracts. The effect was found both with tetraubiquitin and with natural ubiquitinated substrates. The inhibition was due to the ubiquitin binding capacity of the proteins and the ubiquitin binding domains alone were sufficient to protect substrate proteins against deubiquitination. However, Rhp23 and Dph1 appeared to inhibit deubiquitination much more strongly than Pus1 did.

Both Rhp23 and Dph1 bind ubiquitin chains via an $\sim$80 amino acid UBA domain, whereas Pus1 binds via a smaller $\sim$20 amino acid UIM domain. Perhaps due to a steric blockage of the deubiquitinating enzyme the bulky UBA domain in Rhp23 and Dph1 is better suited for inhibition than the smaller UIM domain in Pus1.

Rhp23, Dph1 and Pus1 did not significantly inhibit deubiquitination of ubiquitin-AMC, presumably because they only have very weak affinity for monoubiquitin [10], and therefore also for the ubiquitin-AMC substrate.

Rhp23, Dph1 and Pus1 have been shown to have important functions in the regulation of protein degradation [10], but the biochemical mechanisms which regulate their function remain to be unravelled. The ubiquitination status of a given protein is determined by the balance between ubiquitination and degradation on one hand and deubiquitination on the other. By protecting substrates against deubiquitination, Rhp23, Dph1 and Pus1 may favour degradation rather than deubiquitination, by pushing the equilibrium between degradation and deubiquitination towards degradation. This is a function which only depends on the ability to interact with ubiquitin chains and is therefore likely to be shared by most if not all multiubiquitin binding proteins.

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