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Mutant p62/SQSTM1 UBA domains linked to Paget's disease of bone differ in their abilities to function as stabilization signals

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ABSTRACT

We show that the ubiquitin-associated domain (UBA) of human p62/sequestosome-1 (SQSTM1) can delay degradation of proteasome substrates in yeast. Taking advantage of naturally occurring mutant UBA domains that are linked to Paget's disease of bone (PDB), we found that three of the four mutant UBA domains tested in this study were able to inhibit proteasomal degradation, albeit not to the same extent as the wild-type domain. Interestingly, the stability measured as the fraction of folded protein, and not the ubiquitin binding properties, of the PDB-associated UBA domains correlated with their protective effects. These data suggest that the protective effect of UBA domains depends on their structural integrity rather than ubiquitin binding capabilities.

1. Introduction

A unique and important feature of the ubiquitin/proteasome system (UPS) is its ability to destroy proteins in a spatial and temporal regulated manner by processive degradation [1]. Proteasomal degradation is primarily regulated by means of conjugation of canonical Lys48-linked polyubiquitin chains to substrates [2]. The major determinants of protein turnover are degradation signals, which are the motifs or domains that are recognized by ubiquitin ligases [3]. Although ubiquitylation is intimately linked to proteasomal degradation, it has become apparent that various ubiquitin modifications also play non-proteolytic roles in a number of processes such as transcription and DNA repair [4].

The complex nature of the UPS and the versatile behavior of ubiquitin modifications suggest that additional factors may be involved in the regulation of ubiquitin-dependent proteolysis. Interestingly, the C-terminal ubiquitin-associated (UBA)-2 domain of the DNA repair and ubiquitin shuttling factor Rad23 [5] functions as an intrinsic stabilization signal that prevents degradation of this proteasome-interacting protein [6]. More recently, it was shown that the transcription factor Met4, whose activity is regulated by the conjugation of a Lys48-linked chain [7], was protected from proteasomal degradation by a structurally unrelated ubiquitin interacting motif (UIM) [8]. Biochemical analysis suggested that the protective effect of the UIM was mediated through binding of the UIM domain onto the Lys48-linked polyubiquitin chain on Met4 whereby it prevented the formation of chains that were sufficiently long to target for degradation [8]. Since Rad23's UBA2 and Met4's UIM domains are structurally unrelated but share the ability to bind Lys48-linked polyubiquitin chains, a plausible explanation seems that both domains act through a shared mechanism that relies on ubiquitin binding.

Paget's disease of bone (PDB) is a common chronic disorder that is characterized by a focal increase in bone turnover causing fractures and bone deformations [9]. The etiology of the disease

Abbreviations: CD, circular dichroism; PDB, Paget's disease of bone; SQSTM, sequestosome; UBA, ubiquitin-associated; UIM, ubiquitin interacting motif; UPS, ubiquitin/proteasome system

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is poorly understood but appears to be linked to ubiquitylation [10]. Mutations in the ubiquitin binding protein p62, also known as sequestosome-1 (SQSTM1), have been genetically linked to sporadic and familial cases of PDB [11]. Interestingly, the more than 20 PDB mutations in p62 that have been described are clustered within, or adjacent to, the C-terminal UBA domain [11–16]. In the present study, we have taken advantage of four of these naturally occurring mutant UBA domains and used them to study the role of ubiquitin binding and structural integrity in UBA-mediated protection from proteasomal degradation.

2. Materials and methods

2.1. Yeast strains and media

The experiments were performed with haploid derivates of strain DF5 (lys2-801, leu2-3, -112, ura3-52, his3-Δ200, trp1-1, Mat-α). Yeast transformed with pYES2 plasmids encoding the indicated reporter substrates were grown successively overnight in synthetic minimal medium with a mix of glucose, raffinose, and galactose as sole carbon source until midlog phase. Yeast used in experiments with proteasome inhibitor was grown for 2 h in the presence of 50 μM proteasome inhibitor Z-Leu-Leu-Leu-CHO (MG132; ENZO). To allow uptake of the inhibitor, yeast was grown in synthetic medium with 0.1% proline as sole nitrogen source and 0.003% SDS [17]. Expression from the GAL1 promoter was regulated by using the following mixtures of galactose, raffinose and glucose as carbon sources: 2.0% galactose (high expression), 1.4% galactose and 1.0% raffinose and 0.4% glucose (moderate expression) or 0.8% galactose and 1.0% raffinose and 0.8% glucose (low expression).

2.2. Construction of plasmids

Yeast expression plasmids were generated by PCR amplifying the open reading frames, introducing flanking restriction sites, and cloning them into the yeast expression vector pYES2 Ub-M-GFP and Ub-R-GFP [18]. The UBA domains that were inserted in the fusions compromised the amino acids 388–440 of human p62.

2.3. Protein analysis

Yeast was grown in galactose until midlog phase. Total protein extracts were obtained by lysis of the yeast and precipitation of proteins in trichloroacetic acid. Protein turnover was determined by administration of glucose and cycloheximide (Sigma) to final concentrations of 2% and 1 mg/ml, respectively, to the yeast cultures in midlog phase. Aliquots were taken at the indicated time points and total protein extracts were prepared. Samples were heated at 95 °C and subjected to SDS–PAGE for Western blot analysis.

2.4. Western blot analysis

Total lysates were separated by SDS–PAGE and transferred to nitrocellulose membranes (PROTRAN; Schleicher & Schuell). The membranes were blocked in phosphate-buffered saline supplemented with 5% skim milk and 0.1% Tween-20 and incubated with mixed monoclonal antibody (Roche) or polyclonal antibody (Molecular Probes) specific to GFP. For loading controls, blots were probed with a monoclonal antibody against β-actin (Abcam). After subsequent washing steps and incubation with peroxidase-conjugated goat anti-mouse serum (GE Healthcare) or goat anti-rabbit serum (Jackson ImmunoResearch Laboratories, Inc.), the blots were developed with enhanced chemiluminescence (GE Healthcare).

2.5. Flow cytometric analysis

Yeast was grown in galactose until midlog phase and subjected to flow cytometric analysis on a fluorescence activated cell sorter (FACScalibur; Beckton & Dickinson). The data were analyzed with Cellquest software.

2.6. Circular dichroism

The expression and purification of the domains have been described previously [19,20]. Far UV-circular dichroism (CD) spectra were collected on an Applied PhotoPhysics Pi-star-180 spectrophotometer with a Peltier heating device for melting studies. In equilibrium studies the temperature was regulated using a Neslab RTE-300 circulating programmable water bath and spectra were collected using a 10 mm path length quartz cuvette over a wavelength range from 215–340 nm on 5 μM solutions of protein in 50 mM phosphate buffer at pH 7.0. The melting curves were normalized by converting to the fraction of protein folded as a function of temperature. Lines of best fit to the data represent the least squares fit to a simple two-state unfolding model from which the mid-point of the transition could be determined (T_m).

3. Results

3.1. Wild-type and mutant UBA domains of p62 can function as cis-acting stabilization signals

We first determined whether the wild-type UBA domain of human p62 can inhibit proteasomal degradation. For this purpose, we expressed chimeric proteins that consisted of a proteasome reporter substrate harboring an N-end rule degradation signal and the wild-type or mutant UBA domains at their C terminus (Fig. 1A). As an N-end rule substrate, we used ubiquitin-arginine-green fluorescent protein (Ub-R-GFP) which, upon processing by endogenous deubiquitylation enzymes, exposes a destabilizing N-terminal arginine residue that targets the fusion for ubiquitin-dependent degradation [21]. As a control, we used ubiquitin-methionine-green fluorescent protein (Ub-M-GFP) in which the critical arginine residue had been replaced by a methionine residue and that is hence not targeted for proteasomal degradation. Yeast expressing Ub-R-GFP contained much lower steady state GFP levels than Ub-M-GFP as evidenced by Western blotting (Fig. 1B) and flow cytometry (Fig. 1C), as shown previously [18]. Notably, the Ub-R-GFP levels, but not Ub-M-GFP levels, increased upon treatment with the proteasome inhibitor MG132 consistent with efficient proteasomal degradation of Ub-R-GFP (Fig. 1B).

Introduction of the wild-type UBA domain of p62 in Ub-R-GFP caused a significant increase in the steady state levels of the substrate (Fig. 1B and C), suggesting that this domain can delay proteasomal degradation. All four PDB-associated mutant domains, that were used in this study, have a reduced ability to bind ubiquitin in pull-down assays when present in full length p62 [22]. Isolated recombinant UBA^{M404T} and UBA^{G425R} domains are also severely impaired in ubiquitin binding whereas recombinant UBA^{P392L} and UBA^{G411S} domains are still able to bind ubiquitin comparable to the UBA^{WT} [14]. We found that out of the four mutant domains only the UBA^{M404T} lacked a protective effect (Fig. 1B and C). Introduction of the other three PDB-associated UBA domains, including the UBA^{G425R}, which exhibits severely impaired ubiquitin binding in vitro [19], resulted in a significant increase in the steady state levels of the proteasome substrate although they did not reach the same levels as observed with the wild-type domain. Notably, the UBA^{G425R} domain had a comparable protective effect to the ubiquitin binding competent UBA^{P392L}. These data suggest that

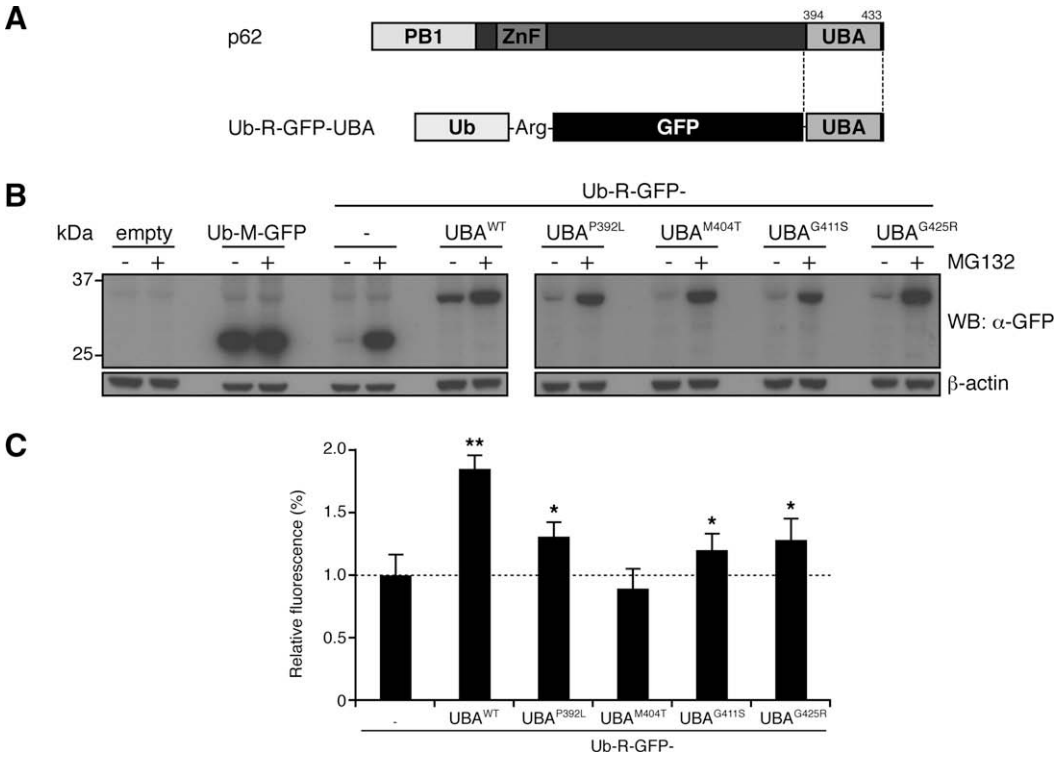


Fig. 1. Wild-type and mutant UBA domains of p62 can function as *cis*-acting stabilization signals. (A) Schematic drawing of the Ub-R-GFP N-end-rule reporter substrate fusion protein and the UBA domain. The location of the UBA domain in p62 is indicated. (B) Steady state levels of Ub-M-GFP, Ub-R-GFP, or the Ub-R-GFP-UBA fusions in yeast in the absence and presence of the proteasome inhibitor MG132. β-Actin is shown as a loading control. (C) Flow cytometric analysis of the GFP fluorescence intensities of yeast expressing Ub-R-GFP and the Ub-R-GFP-UBA fusions. For each construct the intensities of three samples, each consisting of a mixture of three colonies, was determined. The values were standardized to the fluorescence intensity of the Ub-R-GFP expressing strains. Mean and standard deviation are indicated ($n = 6$). Values that were significantly different from Ub-R-GFP are marked. $^{*}P < 0.05$, $^{**}P < 0.01$ (Student's t test).

ubiquitin binding ability is not directly related to the inhibition of proteasomal degradation mediated by UBA domains.

3.2. The UBA-mediated increase in Ub-R-GFP steady state levels is due to prolonged half-lives

We next analyzed the effect of introducing the wild-type or PDB-associated UBA domains on the half-lives of the substrate. While the Ub-R-GFP was degraded within 10 min, the Ub-R-GFP-UBA^{WT} fusion was still present after 40 min (Fig. 2). Also the fusions carrying the UBA^{G425R}, UBA^{P392L} or UBA^{G411S} domains had increased half-lives as compared with the Ub-R-GFP lacking a UBA domain. The UBA^{G425R} domain which is severely impaired in ubiquitin binding and the ubiquitin binding competent UBA^{P392L} had the strongest stabilizing effects of the four mutant domains. The UBA^{G411S} domain gave a slightly weaker stabilization whereas the UBA^{M404T} domain was the only domain that was unable to delay degradation of Ub-R-GFP.

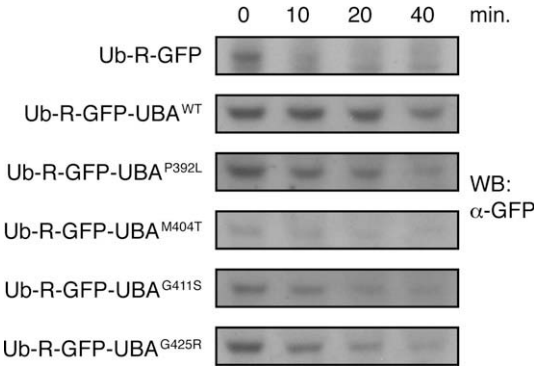


Fig. 2. The UBA-mediated increase in Ub-R-GFP steady state levels is due to prolonged half-lives. Western blot analysis with GFP-specific antibody of turnover of Ub-R-GFP and the Ub-R-GFP fusions in yeast at 0, 10, 20, and 40 min after switching of protein synthesis by administration of glucose and cycloheximide.

3.3. The UBA^{M404T} domain accelerates degradation

We next expressed the fusions in a yeast strain that due to a genetic deletion lacked the Ubr1 ubiquitin ligase responsible for recognition and ubiquitylation of N-end rule substrates [23]. Ub-R-GFP and all fusions with UBA domains except the Ub-R-GFP-UBA^{M404T} fusion were stable in the *ubr1Δ* strain (Fig. 3A). Although the UBA^{M404T} fusion was somewhat stabilized in the *ubr1Δ*, substantial residual degradation remained in the absence of the Ubr1 ubiquitin ligase. Since the relative high expression levels obtained from the GAL1 promoter could mask more subtle destabilizing effects of other mutant UBA domains, we reduced the expression levels of the reporters by growing the yeast in the presence of a mixture galactose, raffinose and glucose (Fig. 3B). Analysis of the turnover of the fusions at this strongly reduced expression level confirmed that only the fusion containing UBA^{M404T} was still degraded in the absence of the Ubr1 ligase (Fig. 3C). These data suggested that the Ub-R-GFP-UBA^{M404T} is targeted for degradation by the mutant UBA domain. Indeed, we observed that insertion of the UBA^{M404T} domain in the stable Ub-M-GFP resulted in proteasomal degradation of this fusion (Fig. 3D). These data show that the UBA^{M404T} domain not only fails to protect the substrate from degradation but accelerates its proteasomal degradation by a mechanism that is independent of the N-end rule degradation signal.

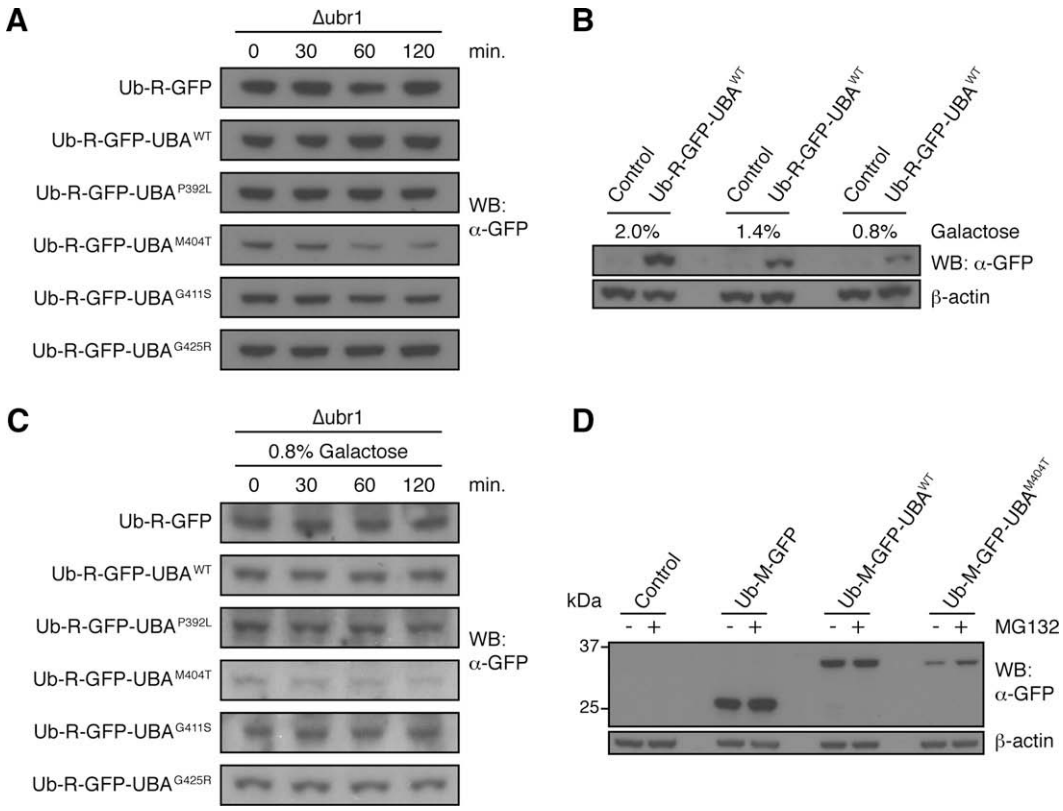


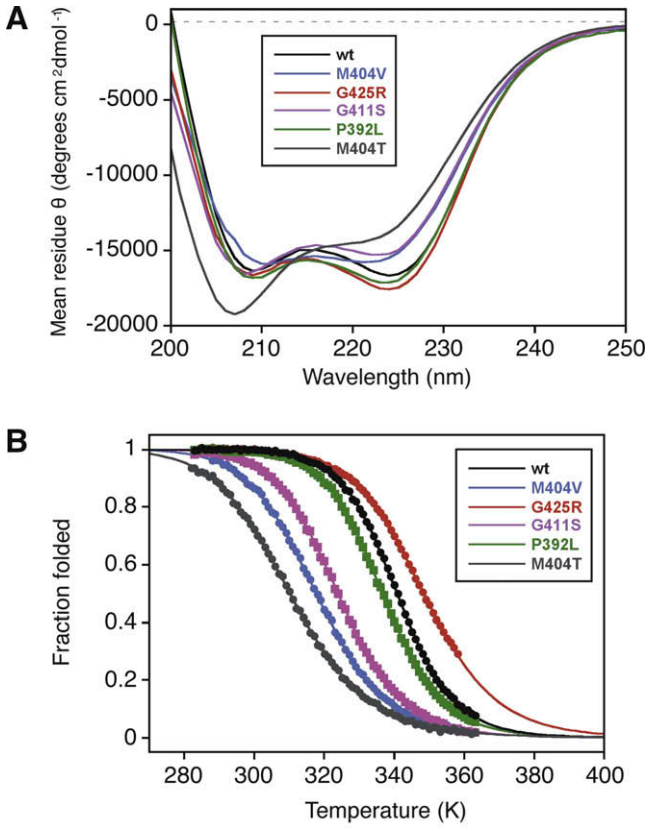
Fig. 3. The UBA^{M404T} domain accelerates degradation. (A) Turnover of the fusion proteins after promoter shutoff in the *ubr1Δ* yeast strain. Samples were collected at the indicated time points after switching of protein synthesis by administration of cycloheximide and analyzed in anti-GFP Western blot analysis. (B) Steady state levels GAL1-driven expression of Ub-R-GFP-UBA^{WT} in *ubr1Δ* yeast grown in the presence of 2.0%, 1.4% or 0.8% galactose. β-Actin is shown as a loading control. (C) Turnover experiment as in described in A but *ubr1Δ* yeast was grown in the presence of 0.8% galactose to reduce the expression level of the fusion proteins. (D) Steady state levels of Ub-M-GFP, Ub-M-GFP-UBA^{WT}, and Ub-M-GFP-UBA^{M404T} in yeast in the absence and presence of proteasome inhibitor determined in a Western blot probed with a GFP-specific antibody. β-Actin is shown as a loading control.

3.4. Thermal stability of PDB-associated UBA domains determined by CD spectroscopy

We investigated the structural integrity and relative stability of the UBA domains using CD spectroscopy. The equilibrium CD spectra were recorded at 298 K using the minimal UBA construct (387–436), except in the case of the UBA^{P392L} mutant which had additional N-terminal and C-terminal sequences (341–440). The UBA^{M404V} PDB mutant was also included in these biophysical analyses, as our turnover experiments indicated M404 to be a critical residue in the UBA domain. The CD spectra are typical of helix-rich proteins with strong minima at 208 and 222 nm (Fig. 4A). The spectra of the mutants generally overlay quite closely with that of the wild-type UBA showing conservation of helical structure. The spectrum of the 341–440 UBA^{P392L} construct gives a greater negative ellipticity at around 207 nm, reflecting the contribution from the extended and flexible N-terminal sequence [19]. We measured the thermal stability of the UBAs from CD melting profiles at protein concentrations of 5 μM (representing the UBA monomer [24]). The introduction of point mutations into the UBA domain produces a significant variation in stability with a 30 K range in T_m values (Fig. 4B). A more useful measure of stability in this context is to describe the fraction of folded protein (Φ_f) at a common temperature. Since the protective effects of the domains were studied at 303 K (30 °C), a physiological temperature in budding yeast, we estimated Φ_f also at this temperature (Fig. 4C). While the UBA^{G425R} and UBA^{P392L} domains were essentially fully folded at this temperature, a significant proportion of the UBA^{G411S} domain was unfolded and the UBA^{M404T} and UBA^{M404V} domains were highly destabilized. We conclude that the protective effect of the mutant UBA domains correlates with their thermal stability and folding state at 30 °C.

4. Discussion

Given the fact that the UIM domain of Met4 displays a protective effect by preventing the formation of polyubiquitin chains that are sufficiently long to facilitate proteasomal degradation [8], it seemed plausible that ubiquitin binding would also play a crucial role in the molecular mechanism for UBA domain-mediated protection [6]. This hypothesis was in particular attractive since the UBA and UIM domains are structurally unrelated but share the ability to bind ubiquitin chains [25]. The observation that the UBA domain of p62 can inhibit proteasomal degradation of reporter substrates has opened the possibility to exploit a number of naturally occurring UBA mutants that exert a range of effects on ubiquitin binding [14,22]. Although this approach revealed that ubiquitin binding activity is not directly related to UBA-mediated inhibition of proteasomal degradation, the data do not exclude a contributing role for ubiquitin binding since all PDB-associated domains were less efficient inhibitors than the wild-type UBA domain. Regardless whether ubiquitin binding or another feature collectively absent in PDB-associated domains contributes to the protective effect, our data unequivocally show that UBA domains can inhibit proteasomal degradation even when their ubiquitin binding activity is severely impaired. This strongly suggests that the molecular mechanism is fundamentally different from that proposed for Met4's UIM domain.



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UBA domain	T_m	Φ_f^{303K}
WT	340.2 K	1.00
P392L	336.7 K	0.99
M404T	310.2 K	0.66
M404V	317.7 K	0.82
G411S	323.9 K	0.92
G425R	348.5 K	1.00

Fig. 4. Thermal stability of PDB-associated UBA domains determined by CD spectroscopy. (A) Equilibrium CD spectra recorded at 298 K for the wild-type UBA and various PDB mutants. (B) Normalised CD melting curves for the UBA domains showing the fraction folded as a function of temperature. The transition mid-point (fraction folded = 0.5) enabled T_m values to be determined. The line of best fit represents the analysis of the transition in terms of a two-state unfolding model. (C) Table showing the estimated T_m values for each UBA domain (error <0.5 K) alongside the fraction of protein folded at 303 K.

This also brings up the question of how the protective effect of the UBA domain is accomplished. Our data show that mutant domains with the greatest protective effects were the most thermally stable indicating that structural integrity may play an important role. In order for proteins to be degraded by the proteasome, the polypeptide has to be unfolded and translocated into the proteolytic chamber of the 20S core particle of the proteasome [26]. This procedure is complex and requires accurate handling of the substrate. Inhibition of a number of events crucial for protein degradation can account for the delayed degradation of UBA domain-containing substrates including (1) substrate recruitment [27], (2) the initiating step of unfolding [28], (3) substrate unfolding [29], (4) the opening of the proteasome gate [30] and (5) substrate translocation [26]. Therefore, further studies are required to empirically probe into the mode of action of UBA domain-mediated stabilization.

Although we cannot formally exclude that this UBA domain prevents proteasomal degradation of p62, this seems to be an unlikely scenario given the biological functions of this protein. A large body of evidence suggests that p62 primarily functions as a scaffolding protein for ubiquitylated proteins in processes that do not implicate proteasomal degradation, such as NF-κB signaling [31] and macro-autophagy [32]. In line with this notion, we did not observe an effect of the PDB-associated UBA domain mutations on p62 turnover in human cells (CB, NPD; unpublished observations), consistent with earlier studies [33]. Thus, our data suggest that p62's UBA domain is unlikely to play a role in protecting this protein from proteasomal degradation although we cannot formally exclude subtle stabilizing effects or the possibility that p62 is only protected from proteasomal degradation under specific conditions. The identification of a protective UBA domain that does not modify the half-lives of its natural host protein suggests that the ability of UBA domains to interfere with proteasomal degradation may be an intrinsic feature of a subclass of UBA domains. Direct comparison of the structural features of protective and non-protective UBA domains may shed light on the molecular mechanisms underlying their inhibition of proteasomal degradation.

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