Regulated association of misfolded endoplasmic reticulum lumenal proteins with P58/DNAJc3

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P58/DNAJc3 defends cells against endoplasmic reticulum (ER) stress. Most P58 molecules are translocated into the ER lumen, and here we report selective and stable binding to misfolded proteins by P58's TPR-containing N-terminal domain. *In vitro*, too, P58 binds selectively to a model misfolded protein and challenge of that complex with physiological concentrations of the ER lumenal Hsp70-type chaperone BiP encourages disassembly. BiP-induced dissociation of P58 from its substrate depends on the presence of ATP and on interactions with P58's J-domain, which are mediated by invariant residues BiP^{R197} and P58^{H422}. A functional J-domain also accelerates dissociation of P58 from a model substrate, VSV-G^{ts045}, on the latter's re-folding *in vivo*. However, J-domain binding can be separated from the ability to promote substrate dissociation by the mutant BiP^{E201G} and a wild-type J-domain fused ectopically to P58^{H422Q} rescues the latter's inability to dissociate from substrate in response to BiP and ATP. These findings are consistent with a model whereby localized activation of the Hsp70-type partner is sufficient to promote substrate handover from the J-domain co-chaperone.

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Introduction

The high flux of newly synthesized (unfolded) proteins through the endoplasmic reticulum (ER) of secretory cells renders the latter especially dependent on chaperones that function in the ER. For this reason, secretory cells have heightened activity of the ER unfolded protein response (UPR), which couples the stress imposed by the load of unfolded proteins in the lumen of the organelle (ER stress) to activation of genes that encode chaperones that function in

the organelle (Schroder and Kaufman, 2005; Bernales *et al*, 2006).

Surprisingly, one of the genes consistently upregulated by the mammalian UPR was predicted to encode a cytoplasmic protein, P58, which was initially identified as an inhibitor of the cytoplasmically localized kinases PKR (Barber *et al*, 1994) and PERK (Yan *et al*, 2002; Van Huizen *et al*, 2003). PERK mediates an adaptive response to ER stress that limits the influx of unfolded proteins into the lumen of the stressed organelle (Ron and Harding, 2007); however, a modulatory function for P58 in the UPR could not readily explain the phenotype of P58 loss of function. In both worms and mice, loss of P58 sensitizes the animals to the consequences of protein misfolding in the ER lumen (Kapulkin *et al*, 2005; Ladiges *et al*, 2005), which is the opposite phenotype of that predicted from the loss of an inhibitor of PERK. Experiments conducted in an effort to explain the P58 knockout phenotype supported a model whereby P58 functions at the cytosolic face of the translocon (Oyadomari *et al*, 2006). However, new findings (Rutkowski *et al*, 2007, confirmed here) place P58 predominantly in the lumen of the ER, and therefore suggest a more direct function for the co-chaperone in buffering the load of unfolded proteins in the ER lumen.

Molecular chaperones form transient complexes with hydrophobic residues exposed on extended segments of non-native substrate proteins. P58 (also known as DnaJc3) contains a J-domain found in a large class of co-chaperones that function alongside the Hsp70 family of chaperones. The ability of Hsp70 to bind substrates is tightly regulated by a nucleotide-binding domain that couples ATPase activity to conformational changes that affect the substrate-binding domain (Szabo *et al*, 1994). In the ATP-bound state, a substrate-binding groove is accessible and substrates associate and dissociate with high 'on/off' rates, whereas the ADP-bound form stably associates with substrates (Schmid *et al*, 1994). J-domain co-chaperones (also called Hsp40s) consist of a diverse family of proteins that share a conserved region of ~70 amino acids, the J-domain, which mediates interactions with partner Hsp70s and markedly stimulates their intrinsically slow rates of ATP hydrolysis (Liberek *et al*, 1991). A conserved motif consisting of His-Pro-Asp (HPD) found in the J-domain is essential for surface interactions with invariant residues on Hsp70, and mutations of the motif abrogate stimulation of Hsp70 ATPase activity (Tsai and Douglas, 1996; Suh *et al*, 1998; Jiang *et al*, 2007).

Several J-domain co-chaperones have been found to bind hydrophobic residue-rich extended segments of unfolded proteins through a separate substrate-interacting domain (Sha *et al*, 2000; Rudiger *et al*, 2001; Li *et al*, 2003) and they are thought to subsequently recruit their Hsp70 partners to that substrate through their J-domain. The current model of sequential binding (first by the co-chaperone and then handover of substrate to Hsp70) is supported by the finding that the J-domain's affinity for Hsp70 is markedly enhanced by the presence of ATP in the latter's nucleotide-binding

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domain (Wawrzynow and Zylicz, 1995). Substrate transfer from the co-chaperone to the chaperone presumably involves J-domain-induced hydrolysis of ATP, and its conversion to ADP, which remains bound. The transfer of substrate is also favoured by the fact that its engagement at the substrate-binding domain of Hsp70 contributes to ATP hydrolysis at the nucleotide-binding domain (Flynn *et al*, 1989; Laufen *et al*, 1999) and the re-cycling of the J-domain co-chaperone is presumably favoured by its disengagement from the ADP-bound Hsp70. However, the function, if any, of the Hsp70 partner in facilitating substrate release from the J-domain co-chaperone has not been addressed. This paper explores the interactions between luminal P58, its substrates and Hsp70 partner, and exploits the surprising stability of the complex between P58 and its misfolded substrate to address the mechanism of substrate release.

Results

Selective binding of misfolded luminal proteins by P58

Consistent with the recent publication of Rutkowski *et al* (2007), we also found that the conserved hydrophobic sequence at P58's N terminus efficiently directs translocation of the protein into the ER lumen and that *in vitro* translated radiolabelled mouse P58 migrated more slowly on SDS-PAGE than endogenous P58 immunopurified from metabolically labelled ER-stressed mouse fibroblasts or from 293T cells transfected with an expression plasmid for the mouse protein (Supplementary Figure S1). These observations are consistent with the presence of a cleavable N-terminal signal sequence in P58 that directs most of the P58 synthesized in cells into the ER lumen.

P58's localization to the ER lumen suggests that its ability to ameliorate the consequences of ER stress might stem from interactions with misfolded luminal ER proteins. To explore this possibility, Chinese hamster ovary (CHO) cells were stably transduced with a gene encoding mouse P58 modified to contain a cleavable signal sequence followed by an eight-residue FLAG epitope tag that is recognized by the FLAG-M1 monoclonal antibody only in the context of the protein's N terminus (which is exposed only after cleavage of the signal peptide; Supplementary Figure S2). Immunoprecipitation with the FLAG-M1 antibody thus confines the analysis to luminal P58 and also overcomes the challenge posed by the fact that none of the antibodies or antisera to P58 in our possession immunoprecipitates the protein under native conditions.

FLAG-P58 recovered by immunopurification from CHO cells was stably associated with a co-expressed misfolding-prone fusion between mutant insulin (INS2^{C96Y}) and green fluorescent protein (GFP) but not with the more abundant co-expressed cytosolic GFP (Supplementary Figure S3). To explore in more detail this suggestion of an interaction of P58 with misfolded proteins, we turned to a well-characterized ER substrate, the folded state of which can be reversibly manipulated by temperature shift of expressing cells. The *ts045* mutation in the extracellular/luminal portion of the vesicular stomatitis virus envelope glycoprotein (VSV-G^{ts045}) causes misfolding and ER retention at the non-permissive temperature of 41°C but is compatible with normal folding and ER exit at the permissive temperature of 32°C (Kreis and Lodish, 1986).

VSV-G^{ts045}, tagged at its cytoplasmic portion by GFP, was expressed transiently in the CHO cells stably expressing FLAG-P58. Immunoblotting of FLAG-M1 immunoprecipitates with a GFP antiserum (that detects VSV-G^{ts045}-GFP) revealed a small amount of VSV-G^{ts045}-GFP in complex with P58 at steady state in all culture conditions (Figure 1A), but this increased by culture at the non-permissive temperature, especially in the presence of the proteasome inhibitor MG132 (Figure 1A, lane 4), which presumably stabilizes VSV-G^{ts045}-GFP (whose half-life in the FLAG-P58 cells is rather short, ~0.5 h; Supplementary Figure S4). Significantly, shift of cells cultured at the non-permissive temperature back to the permissive temperature led to dissociation of much of the complex within 1 h (Figure 1A, compare lanes 4 and 6).

These experiments suggest a dynamic association of P58 and misfolded VSV-G, which could be direct or indirect. Furthermore, as folding (at the permissive temperature) leads to exit from the ER, the above experiment does not tell us whether P58 discriminates between ER-localized folded and misfolded VSV-G. To uncouple folding from ER exit, we used the drug brefeldin A (BFA), which blocks exit of all proteins (folded and unfolded) from the ER (Misumi *et al*, 1986). To confirm retention of VSV-G^{ts045}-GFP in the ER of cells exposed to BFA, the protein was immunoprecipitated with the anti-GFP serum from cells that were metabolically labelled at the permissive or non-permissive temperature in the absence or presence of BFA. To monitor ER exit, the immunopurified proteins were subjected to endoglycosidase H (Endo H), an enzyme that selectively removes N-linked glycans that had not undergone modification by Golgi enzymes (Trimble *et al*, 1978). VSV-G^{ts045}-GFP synthesized at the permissive temperature (in the absence of BFA) is mostly Endo H resistant and migrates slowly on SDS-PAGE; the slow mobility presumably reflects further Golgi modifications of N-linked glycans. Inhibition of ER exit by BFA sensitizes the protein to Endo H and enhances its mobility on SDS-PAGE (Figure 1B, lanes 1–4). By contrast, VSV-G^{ts045}-GFP synthesized at the non-permissive temperature is mostly Endo H sensitive and addition of BFA effects a slight (paradoxical) increase in the fraction of the protein that is Endo H resistant; presumably by promoting exposure of misfolded, ER-localized VSV-G^{ts045}-GFP to Golgi-modifying enzymes from the collapsed Golgi apparatus (in the BFA-treated cells) (Figure 1B, lanes 5–8).

Having unlinked VSV-G^{ts045}-GFP folding from ER exit it was then possible to inquire as to the role of VSV-G's misfolding in its association with P58. FLAG-P58 CHO cells transiently expressing VSV-G^{ts045}-GFP were metabolically labelled at the permissive or non-permissive temperature in the presence of BFA, and radiolabelled proteins in complex with P58 (immunopurified with FLAG-M1) were revealed by SDS-PAGE and autoradiography. Labelled VSV-G^{ts045}-GFP was recovered in complex with P58 only in cells labelled at the non-permissive temperature (Figure 1C). The effectiveness of BFA in retaining the correctly folded VSV-G^{ts045}-GFP in the ER (at the permissive temperature) is revealed by the identical mobility of the protein extracted from cells labelled (and chased) at the permissive and non-permissive temperature (lanes 5–8).

To expand on these observations, we metabolically labelled cells at the non-permissive temperature and then either left them at the non-permissive or shifted them to the

permissive temperature during a non-labelled ('cold') chase period. The complex of P58 and VSV-G^{ts045}-GFP, which is stable for 1 h of chase at the non-permissive temperature, dissociated over a similar period of chase at the permissive temperature (Figure 1D, compare lanes 4 and 5). These experiments reveal that P58 selectively associates with misfolded VSV-G and that the latter is released from the complex on folding.

A 78-kDa protein, which is likely to be the abundant ER chaperone BiP, is a reproducible constituent of the P58-containing complexes (Figures 1C and D, and 2; Rutkowski

et al, 2007). To determine whether BiP mediates association of VSV-G^{ts045}-GFP with P58, we exposed FLAG-P58 immunopurified complexes (from cells metabolically labelled at the non-permissive temperature) to ATP and Mg²⁺. As predicted, this led to dissociation of BiP from the complex but had only a minor effect on the content of misfolded VSV-G (Figure 2A and B, compare lanes 3 and 4 in each).

BiP-independent association of P58 with misfolded VSV-G^{ts045}-GFP suggests the possibility of direct binding of misfolded proteins by P58. To explore this possibility, we exposed lysates from mammalian cells expressing P58 to a sepharose resin containing covalently coupled native bovine RNase A or an identical resin on which the RNase A had been reduced and denatured (see Materials and methods section). More P58 was retained on the resin containing denatured RNase A than on the resin containing native RNase A; denaturation of some RNase A molecules during the cross-linking procedure might contribute to the residual binding of P58 to the native resin (Figure 3A, lanes 3 and 6). Endogenous BiP from the cell lysates associated selectively with the denatured bait, but unlike P58, dissociated from the resin in the presence of ATP and Mg²⁺. This experiment indicates that P58 distinguishes between a folded and misfolded version of the same protein. A C-terminal truncated P58 lacking its J-domain also bound selectively to the denatured protein, indicating that the J-domain is dispensable to selective binding and the TPR motif-containing N-terminus of P58 is sufficient for interaction with substrate (Figure 3A, lanes 6–8).

To gain further insight into the interaction between P58 and misfolded proteins, we measured the ability of a cross-linking reagent to covalently link P58 and denatured RNase A. A complex between P58 from mammalian cell lysates and the denatured RNase A-containing affinity resin was exposed

Figure 1 P58 selectively associates with misfolded VSV-G *in vivo*. (A) Immunoblot of GFP-tagged VSV-G^{ts045} recovered in complex with FLAG-P58 from lysates of FLAG-P58-expressing CHO cells that had been maintained at the permissive temperature (32°C) or the non-permissive temperature (41°C) for 12 h before harvest or switched back to the permissive temperature for the last hour before harvest (41 → 32°C). Where indicated, the cells were exposed to the proteasome inhibitor MG132 for 3 h before harvest. The middle panel reports on the amount of VSV-G^{ts045}-GFP in the cell lysates (1.5% of the input) and the lower panel on the recovery of FLAG-P58 in the immunoprecipitate. (B) Autoradiograph of immunopurified VSV-G^{ts045}-GFP from transfected CHO cells metabolically labelled for 30 min at the permissive (32°C) or non-permissive temperature (41°C) in the absence or presence of brefeldin A (BFA). Where indicated, the immunopurified proteins were digested by an endoglycosidase that does not react with Golgi-modified N-linked saccharides (Endo H). The slower migrating Endo H-resistant (Endo H^R) and faster migrating Endo H-sensitive (Endo H^S) forms of VSV-G-GFP are indicated. (C) Autoradiograph of proteins immunopurified from CHO cells co-expressing FLAG-P58 and VSV-G^{ts045}-GFP. The cells were briefly radiolabelled (30 min) in the presence of BFA at the permissive (32°C) or non-permissive temperature (41°C) and where indicated, chased with unlabelled media for 30 min in the continued presence of BFA, before lysis and immunoprecipitation with anti-FLAG antibody or anti-GFP serum. (D) Same as (C) except that cells were pulse labelled in the presence of BFA at the non-permissive temperature (41°C) and a cold chase was performed for 1 h at the permissive (32°C, lane 4) or non-permissive temperature (41°C, lane 5) in the continued presence of BFA. The lysate of cells lacking FLAG-P58 (CHO parental, lanes 1 and 6) serves as a specificity control for the recovery of VSV-G^{ts045}-GFP in complex with FLAG-P58.

Figure 2 The complex between P58 and misfolded VSV-G is independent of BiP. (A) Autoradiograph of proteins immunopurified from CHO cells expressing VSV-G^{ts045}-GFP and FLAG-P58 (lanes 1-4) or control, parental CHO cells (lanes 5 and 6), metabolically labelled at the non-permissive temperature. Where indicated, the immunopurified FLAG-P58-containing complex was challenged by incubation at room temperature (25°C) in the presence or absence of ATP (to dissociate bound BiP). The VSV-G^{ts045}-GFP, BiP and FLAG-P58 that remained associated with the immune complex are revealed by autoradiography. (B) BiP and FLAG immunoblot of material prepared as in (A), except that lane 1 contains 1% of the input of the lysate used in lanes 2-4.

to DSP, a crosslinker with a 12-Å spacer arm containing a disulphide bond that can be cleaved under reducing conditions. Non-covalently bound P58 was readily eluted by exposing the resin-bound complex to 4% SDS. P58 that remained associated with the resin by the DSP-induced crosslinks was next eluted with 100 mM DTT. Addition of crosslinker reduced the amount of P58 recovered in the first elution (with 4% SDS, which only disrupts non-covalent interactions, Figure 3B, compare lanes 3 and 4) and led to the retention of P58 as a covalently bound complex with denatured RNase A that was recovered in the second elution (with DTT, which cleaves the crosslinker; Figure 3B, compare lanes 6 and 7). Addition of ATP and Mg^{2+} to the pre-formed complex (and washing the eluted proteins away before adding crosslinker) did not affect the amount of P58 crosslinked to the misfolded RNase A resin, but, as expected, led to BiP's dissociation from the resin and markedly diminished the amount of BiP that was crosslinked (lower panel, compare lanes 4, 5 and 7, 8). P58 lacking the J-domain also bound and crosslinked to the denatured RNase A (lanes 9–14). To determine whether other components in the mammalian cell lysate that were retained on the resin contribute to the specificity of this interaction, we first purified FLAG-P58 from CHO cell lysates by binding it in high salt to a FLAG affinity resin and eluted the bound FLAG-P58 by FLAG peptide competition (Figure 3C). The purified FLAG-P58

Figure 3 Direct binding of P58 to misfolded RNase A *in vitro*. (A) Immunoblots of full-length FLAG-P58 (P58^{WT}) and C-terminally truncated FLAG-P58 (P58^{Δ5}) from lysates of transfected 293T cells associated with an affinity resin consisting of native (N) or denatured (D) RNase A coupled covalently to sepharose beads. The resin was reacted with cell lysates, washed, followed by incubation in the presence of ATP where indicated, further washed and the remaining bound material was eluted and revealed by SDS-PAGE. Lanes 1 and 2 report on the input of the binding reactions. The lower panel (a BiP immunoblot) reports on the association of endogenous BiP with the resin. (B) As in (A), except that the crosslinker DSP was added to the assembled complex followed by sequential elution (indicated by the arrow), first with SDS (to recover non-covalently bound proteins) and then with DTT (to reverse the crosslink and recover covalently bound proteins). Where indicated, the pre-formed complex was reacted with ATP and washed extensively *before* adding cross linker. (C) Coomassie-stained gel of wild-type FLAG-P58 purified from stably expressing CHO cells by FLAG immuno-affinity chromatography and elution by FLAG peptide competition. The crude lysate (input), the column flow-through (FT) and the first and second elution by FLAG peptide competition elution are shown (E1 and E2). (D) Immunoblot of the purified FLAG-P58 (shown in (C)) that associated with the native (N) and denatured (D) RNase A affinity resin. Where indicated, the complex was challenged with ATP. Lane 1 shows 5% of the input used in the other lanes.

also exhibited preference for binding denatured over native RNase A and binding was indifferent to ATP and Mg^{2+} (Figure 3D).

BiP- and ATP-dependent dissociation of P58 from substrates

The above observations are consistent with direct binding of P58 to misfolded proteins and suggest that the N-terminal portion of the co-chaperone mediates such binding. However, the presence of a functional J-domain in P58 and physical evidence for its association with BiP (Rutkowski *et al*, 2007) suggest that the two proteins might cooperate in coping with misfolded proteins. Addition of bacterially expressed P58 to BiP did not enhance the latter's binding to misfolded proteins *in vitro* (data not shown). However, we noted that challenge of pre-existing complexes of purified FLAG-P58 and denatured RNase A with purified bacterially expressed BiP led to substantial dissociation of P58 from the complex in the presence of ATP and Mg^{2+} (Figure 4A, lanes 1 and 2). The effect was dependent on a functional interaction with the J-domain and was abolished by a mutation BiP^{R197E} (Awad *et al*, 2008), in an invariant arginine known to mediate interactions of Hsp70 chaperones with their J-domain co-chaperones (Suh *et al*, 1998; Jiang *et al*, 2007) (Figure 4A, lanes 3 and 4). The enhanced apparent mobility of the residual FLAG-P58 (Figure 4A, lanes 1, 3 and 4) reflects a reproducible SDS-PAGE compression artefact caused by large amounts of BiP bound to the resin, whereas the enhanced binding of BiP^{R197E} to the resin in the presence of ATP and Mg^{2+} (Figure 4A, lane 4) is a reproducible finding explained by the effect of the mutation on the functional coupling of nucleotide and substrate binding by BiP (Awad *et al*, 2008). BiP-dependent dissociation was similarly observed when the complex between P58 and denatured RNase A was formed in a crude lysate of cells expressing the various proteins, circumventing the need to extensively purify P58 (Figure 4B, lanes 4 and 5). In this simple assay, the effect of ATP and Mg^{2+} was dependent on the concentration of BiP (Supplementary Figure S5) and was abolished both by truncation of BiP's peptide-binding domain (BiP-44K) and by BiP^{R197E} (Figure 4B, lanes 6–9).

A role for J-domain interaction with BiP in mediating this ATP/ Mg^{2+} -dependent dissociation is further supported by the finding that the association of C-terminally truncated P58 (P58^{ΔJ}) with denatured RNase A was unaffected by the addition of BiP and ATP/ Mg^{2+} (Figure 4B, lanes 10–13). This conclusion is also supported by the finding that a mutation, P58^{H422Q}, in an invariant histidine that mediates J-domain interactions with Hsp70 chaperones (Suh *et al*, 1998), also abolishes the ability of BiP and ATP/ Mg^{2+} to dissociate the complex between P58 and denatured RNase A (Figure 4C). A role for J-domain-mediated interactions between chaperone and co-chaperone is also supported by the finding that BiP that had bound substrate independently of P58 (i.e. in the presence of ADP) is unable to effect the latter's release when later challenged with ATP (data not shown).

The findings noted above implicate an interaction between BiP and the J-domain of P58 in promoting the latter's dissociation from its substrates *in vitro*. To determine whether an *in vivo* correlate of this phenomenon might also exist, we compared the stability of VSV-G binding to wild-type and the J-domain mutant P58^{H422Q}, which is unable to interact with BiP (see below). CHO cells, expressing similar levels of FLAG-P58^{WT} and FLAG-P58^{H422Q}, were transfected with VSV-G^{ts045}-GFP, and radiolabelled proteins associated with FLAG-P58 were recovered by immunoprecipitation at

Figure 4 Addition of BiP and ATP dissociates P58 from misfolded RNase A. (A) Immunoblot of purified FLAG-P58 (shown in Figure 3C and D) associated with denatured RNase A. Where indicated, the pre-formed complex was challenged with 9 μ M bacterially expressed wild-type BiP (WT) or mutant BiP^{R197E} in the presence of ADP or ATP. The ratio of P58 retained on the resin was measured by the LI-COR IR imaging system and is indicated for each experimental pair (under the curved arrow). The bound BiP was revealed by staining the blot with Ponceau S (lower panel). (B) As in (A) but using wild-type FLAG-P58 (WT) and a C-terminal truncation mutant FLAG-P58 lacking the J-domain (Δ J) from mammalian cell lysates associated with denatured RNase A. Where indicated, the pre-formed complex was challenged with 9 μ M wild-type BiP (WT), BiP lacking its C-terminal substrate-binding domain (44K) and mutant BiP^{R197E}. The BiP that remained bound to the resin is revealed in this experiment as a residual fluorescent signal on the P58 immunoblot. (C) Same as (B), but using mammalian cell lysates expressing mutant FLAG-P58^{H422Q}.

the end of a 30' labelling pulse conducted at the non-permissive temperature (that promotes VSV-G^{ts045}-GFP misfolding) or following a chase at the permissive temperature (that promotes re-folding). The complex between P58^{WT} and VSV-G^{ts045}-GFP decayed reproducibly faster than the complex with P58^{H422Q} (Figure 5), an observation similar to that reported for ERdj3 (Shen and Hendershot, 2005) and consistent with a role for BiP in promoting the J-domain-dependent dissociation of P58 from its substrate.

To gain further insight into the molecular mechanisms involved in BiP-induced dissociation of P58 from substrates, we sought to determine whether the isolated J-domain of P58 could compensate (*in trans*) for BiP's inability to dissociate a

Figure 5 A J-domain mutation that compromises interaction with BiP stabilizes P58's binding to VSV-G^{ts045}-GFP in cells. (A) Autoradiograph of ³⁵S-radiolabelled proteins recovered in an anti-FLAG-M1 immunoprecipitate from lysates of CHO cells stably expressing wild-type P58 or the H422Q J-domain mutant. The cells were transfected with a VSV-G^{ts045}-GFP expression plasmid, pulsed at the non-permissive temperature (41°C) and chased for the indicated time at the permissive temperatures, before isolation of the immune complexes. The location of the radiolabelled P58 and VSV-G is indicated. (B) Autoradiograph of ³⁵S-radiolabelled VSV-G-GFP recovered by immunoprecipitation with anti-GFP serum from the flow through of the reactions shown in (A). (C) Immunoblot comparing the steady-state levels of wild-type and H422Q mutant FLAG-P58 in the CHO cells. (D) Plot of the signal of labelled VSV-G bound to P58 from (A). CHO cells stably expressing FLAG-P58^{WT} (▲) and CHO cells stably expressing FLAG-P58^{H422Q} (■).

J-domain mutant P58^{H422Q} from denatured RNase A. Complexes of unfolded RNase A and the J-domain mutant P58^{H422Q} were assembled *in vitro* and challenged with BiP and ATP. As expected, the mutation in P58^{H422Q} prevented ATP-mediated complex disassembly (Figure 6A, lanes 3 and 4). Addition of a purified fusion protein of GST with P58's isolated J-domain (residues 384–470) (GST-J^{WT}) had no reproducible effect on the stability of complexes of denatured RNase A and mutant P58^{H422Q} (lane 5). By contrast, when added to complexes of denatured RNase A and P58^{WT}, the isolated J-domain of P58 (GST-J^{WT}) reproducibly attenuated the dissociation by BiP and ATP (lanes 6–8). The ability of

GST-J^{WT} to interfere with complex disassembly by BiP was dependent on the functionality of the J-domain, as it was reversed by the H422Q mutation (compare lanes 11 and 12). Functionality of the GST-J^{WT} fusion protein used in these assays was revealed by its ability to bind BiP in an ATP-dependent manner (Figure 6B) and by its ability to promote BiP-dependent ATP hydrolysis (Figure 6C), assays in which the GST-J^{H422Q} mutant proved inert, as expected. These experiments are most consistent with a model whereby the ability of nucleotide-bound BiP to promote dissociation of complexes between misfolded substrates and P58 requires an interaction with the J-domain of the substrate-bound P58 molecule.

The observations noted above are consistent with both an allosteric model, whereby engagement of P58's J-domain by BiP reduces P58's affinity for substrate, and a kinetic model, whereby the engagement of BiP by P58's J-domain affects BiP's affinity for substrate. To help discriminate between these possibilities, we sought to separate BiP-J-domain interaction from complex disassembly. The mutant BiP^{E201G} bound the isolated J-domain of P58 (GST-J^{WT}) (Figure 7A), but failed to dissociate P58 from substrate (Figure 7B, lanes 4–7), thus separating J-engagement from complex disassembly. The E201G mutation also abolishes BiP's basal (Gaut and Hendershot, 1993) and J-domain-stimulated ATPase activity (Figure 7C) and corrupts the nucleotide dependence of J-binding; unlike wild-type BiP that binds J-domains only in the presence of ATP, J-binding by BiP^{E201G} occurred in the presence of either ADP or ATP (Figure 7A, lanes 8–11). Therefore, the mutation does not discriminate definitively between an anomalous mode of J-domain engagement (that impedes allosteric regulation of P58 by the mutant BiP) from inability of the mutant BiP to complete a cycle of ATP hydrolysis and thereby modulate its affinity for substrate to effect the latter's kinetic partitioning away from P58.

To try and de-convolute this issue, we made a V461F mutation in BiP's substrate-binding site (analogous to the V436F mutation in DnaK; Laufen *et al*, 1999), which compromised both BiP's ability to interact with substrate and to promote dissociation of P58 from substrate (Supplementary Figure S6A). P58's J-domain stimulated ATP hydrolysis by BiP^{V461F}, suggesting that BiP^{V461F} interacts productively with P58-J and supporting a role for substrate binding by BiP in the dissociation reaction (Supplementary Figure S6B). Instability of the complex between BiP^{V461F} and P58-J (Supplementary Figure S6C) seemingly weakens this conclusion, by suggesting that the BiP^{V461F} mutation also affects interactions between BiP's NBD and the P58-J. However, this caveat is mitigated by BiP^{V461F}'s inability to bind unfolded substrates and by the known requirement for the immobilized J-protein to serve as a substrate in retaining steady-state binding of Hsp70 (Mayer *et al*, 1999).

To convey the conformational changes from the J-domain to P58's substrate-binding domain, an allosteric mechanism, if it exists, would likely constrain the relationship between P58's functional domains. Therefore, we tested the ability of an ectopic J-domain fused to the N terminus of a mutant P58^{H422Q} to rescue the latter's inability to undergo BiP/ATP-mediated dissociation from its substrate. The J^{WT}-P58^{H422Q}, J^{H422Q}-P58^{H422Q} fusion proteins and the parental P58^{WT} purified from *Escherichia coli* exhibited the expected specificity for denatured over native RNase A (Figure 8A).

Figure 6 Addition of purified P58 J-domain compromises BiP's ability to promote dissociation of P58 from denatured RNase A. (A) Immunoblot of FLAG-P58^{WT} and a J-domain mutant FLAG-P58^{H422Q} from mammalian cell lysates associated with denatured RNase A. Where indicated, the pre-formed complex was challenged with bacterially expressed BiP (9 μ M) in the presence of ADP or ATP and the presence or absence of equimolar wild-type or mutant versions of P58's isolated J-domain (residues 384–470) expressed as a fusion protein with GST (GST-J^{WT} and GST-J^{H422Q}). The amount of P58 retained on the resin following each manipulation was measured by the LI-COR IR imaging system and is indicated (as a percentage of the signal in the unmanipulated sample). BiP's association with the resin is revealed by Ponceau S staining of the blot (lower panel). (B) Coomassie-stained SDS-PAGE of BiP retained on glutathione sepharose beads to which GST, GST-J^{WT} or GST-J^{H422Q} had been pre-bound. Where indicated, the binding buffer was supplemented with ADP or ATP. Lanes 1–4 report on the input proteins used in the experimental lanes 5–10. (C) ATPase activity of BiP alone or in the presence of GST-J^{WT} or GST-J^{H422Q}, measured by the conversion of ATP to ADP. The mean $\pm$ s.e.m. of the fluorescence polarization signal of AlexaFluor 633-labelled ADP tracer, which is dissociated from an anti-ADP antibody in the presence of ADP generated in the reaction from BiP's ATPase activity, is shown ($n = 3$). The fluorescent polarization signal is inversely proportional to the ATPase activity.

The ectopic wild-type J-domain (but not the H422Q mutant, which serves as a specificity control) rescued the inability of P58^{H422Q} to dissociate from substrate in the presence of BiP/ATP (Figure 8B, lanes 6–9). Interestingly, the presence of an ectopic wild-type J-domain at the N terminus of P58^{H422Q} also enabled BiP/ATP to promote dissociation of C-terminal truncations of J^{WT}-P58^{H422Q} from substrate, arguing that linkage of the J-domain to the substrate-binding domain is sufficient to mediate the effect. These findings reveal the portability of P58's J-domain and argue against a constrained relationship between P58's functional domains that is predicted by an allosteric mechanism for substrate release.

Discussion

This study confirms that P58, a protein previously believed to reside in the cytosol, possesses a cleavable N-terminal signal

peptide that mediates translocation into the ER lumen. Most of the signal detected in immunoprecipitates of radiolabelled endogenous P58 migrates on SDS-PAGE as a single band of higher mobility than the *in vitro* translation product of the P58 mRNA, suggesting that the signal peptide is cleaved in most P58 molecules in cultured fibroblasts. These observations, together with those reported in a detailed study (Rutkowski *et al*, 2007), suggest that most of the P58 are located in the ER lumen. Although the new findings do not rule out a role for small amounts of cytoplasmic P58 in modulating the activity of the cytoplasmically located kinase domain of PERK (Yan *et al*, 2002) or in interacting with proteins that are delayed at the ER translocon (Oyadomari *et al*, 2006), they behoove us to consider alternative roles for P58. The evidence from this study, of a direct interaction between P58 and misfolded proteins suggests a simple model whereby P58 assists in the handling of unfolded or misfolded

Figure 7 The BiP^{E201G} mutation separates J-domain binding from dissociation of P58 from denatured RNase A. (A) Coomassie-stained SDS-PAGE of BiP or BiP^{E201G} mutant retained on glutathione sepharose beads to which GST, GST-J^{WT} or GST-J^{H422Q} had been pre-bound. Where indicated, the binding buffer was supplemented with ADP or ATP. Lanes 1–5 report on the input proteins used in the experimental lanes 6–13. (B) Immunoblot of bacterially expressed and purified P58 associated with denatured RNase A. Where indicated, the pre-formed complex was challenged with bacterially expressed BiP^{WT} or BiP^{E201G} (9 μ M) in the presence of ADP or ATP. (C) ATPase activity of wild-type BiP and the E201G mutant alone or in the presence of GST-J^{WT} or GST-J^{H422Q}, measured by the conversion of ATP to ADP. The mean $\pm$ s.e.m. of the fluorescence polarization signal is shown (as in Figure 6C).

luminal proteins as a luminal J-domain containing co-chaperone.

ER homeostasis is dependent on the organelle's ability to rid itself of terminally misfolded/misassembled proteins. Chaperones contribute to this process, presumably by binding to the misfolded proteins and retaining them in a retro-translocation-competent state (Tsai *et al*, 2002). We have observed no reproducible effect of modulating P58 levels on the degradation of a model misfolded secreted glycoprotein (mutant α 1-antitrypsin), a misassembled membrane glycoprotein (T-cell receptor α -subunit) (Oyadomari *et al*,

Figure 8 Ectopic fusion of the J-domain to the N terminus of P58^{H422Q} rescues the protein's inability to dissociate from misfolded RNase A in response to BiP/ATP. (A) Coomassie-stained gel of bacterially expressed proteins associated with the native (N) and denatured (D) RNase A affinity resin. The resin was reacted with purified bacterially expressed P58, or fusion proteins consisting of P58's J-domain (either wild-type or H422Q) fused ectopically to P58^{H422Q}'s N terminus, washed and the remaining proteins were eluted in SDS. Lanes 1–3 contain 10% of the input used in the respective binding reactions. The migration of P58, J-P58 fusion proteins and their N-terminal fragments are indicated. (B) Immunoblot of material as in (A). Where indicated, the pre-formed complex with denatured RNase A was challenged with bacterially expressed BiP^{WT} (9 μ M) in the presence of ADP or ATP. The amount of P58 retained on the resin following each manipulation was measured by immunoblot with a LI-COR IR imaging system and is indicated (as a percentage of the signal in the unmanipulated sample). Note that the N-terminal fragments of J^{WT}-P58^{H422Q} (but not those of the J^{H422Q}-P58^{H422Q} control) were also dissociated by BiP/ATP.

2006), a misfolded membrane-associated glycoprotein (VSV-C^{ts045}-GFP; Supplementary Figure S3) or a misfolded secreted non-glycoprotein (a fusion of mutant pro-INS2^{C96Y} and GFP, data not shown). These negative results suggest that P58 does not have a pervasive role in clearing the ER of misfolded proteins by promoting their degradation. Instead, P58 may reduce the burden of unfolded and misfolded proteins by promoting protein folding in the ER lumen. Direct binding of unfolded/misfolded luminal proteins by P58 explains previous observations that more newly synthesized (metabolically labelled) proteins can be crosslinked to P58 in stressed cells (Oyadomari *et al*, 2006).

J-domain co-chaperones contribute to protein folding by collaborating with chaperones of the DnaK/Hsp70 class (Craig *et al*, 2006). J-domain co-chaperone interactions with unfolded substrate proteins are believed to precede binding to the Hsp70 partner. By promoting nucleotide hydrolysis in the latter, J-domain co-chaperones affect an allosteric shift that increases the affinity of the Hsp70 partner for the unfolded protein and promotes stable Hsp70-substrate protein interaction. Most J-domain co-chaperones interact transiently

with the unfolded substrate and the handover of the substrate from the J-domain co-chaperone to the Hsp70 partner is envisioned as being driven by mass action (Bukau *et al*, 2006). P58 seems unusual in that regard, as the complex it forms with unfolded proteins is quite stable (at least *in vitro* where it survives extensive washing).

We find that physiological concentrations of the abundant ER Hsp70-type chaperone, BiP, can promote dissociation of the P58–substrate complex *in vitro*. However, this effect requires ATP and is sensitive to mutations in either partner that disrupt J-domain–BiP interactions. Taken together, these observations suggest that a productive interaction between ATP-bound BiP and P58, mediated through the latter's J-domain, is required for the dissociation of P58 from its misfolded substrate. A similar phenomenon is observed in ERdj3's interactions with BiP (Jin *et al*, 2008), suggesting that reciprocal regulation of co-chaperone binding to substrate by the Hsp70 partner might be a conserved feature of this chaperone network.

Our findings could be explained by simple competition between BiP and P58 for binding sites on the unfolded substrate, leading to P58's displacement by mass action at steady state. This model is disfavoured by the observation that conditions leading to disruption of the P58–substrate complex (i.e. ATP/Mg²⁺) are associated with less binding of BiP to the denatured RNase A (the substrate) and by the finding that more mutant BiP^{R197E}, which is unable to direct the dissociation of P58, is bound to the substrate than wild-type BiP.

The failure of the isolated J-domain of P58 to complement the BiP/ATP-mediated dissociation of the mutant P58^{H422Q} from its unfolded substrate argues that nucleotide-bound BiP must interact with the J-domain of substrate-bound molecules of P58 for the latter's release to take place. This requirement is consistent with allosteric regulation of P58 by BiP through the J-domain. Several findings argue against this possibility: the three types of J-co-chaperones (I–III) (Cheetham and Caplan, 1998) vary considerably in the arrangement of their J-domain relative to the substrate-binding domain, which seems incompatible with a common allosteric mechanism. The portability of P58's J-domain (Figure 8) also argues against allostery, as the latter is predicted to constrain the relationship of the two domains. BiP's ability to promote dissociation of N-terminal fragments of J^{WT}–P58^{H422Q} provides further evidence against the constraints predicted by the allosteric model; though of course it is impossible to exclude the fortuitous re-creation of a functional machine in the J^{WT}–P58^{H422Q} fusion protein used to test this hypothesis. Finally, the separation of J-domain interaction from substrate release by BiP^{E201G} and BiP^{V461F} argues against allostery. However, it remains formally possible that the mutations in BiP, though permissive for J-domain interactions, selectively corrupt the allosteric mechanism.

More plausible, in our opinion, is an alternative model whereby the J-domain promotes cycles of competitive binding by BiP in proximity to the co-chaperone, followed by dissociation of both partners. The argument for proximity is supported by the observation that the isolated wild-type P58 J-domain (but not the BiP-binding defective mutant J^{H422Q}) interferes (*in trans*) with BiP/ATP-mediated dissociation of P58 from its substrate. Local competition and kinetic partitioning of the substrate from the J-domain co-chaperone to

the Hsp70 partner likely involve ATP binding, hydrolysis and nucleotide exchange by the latter. Although global refolding of the substrate is precluded in our experiments by blocking disulphide-bond formation, local remodelling of the substrate by BiP remains possible. The inability of BiP^{E201G} to properly handle nucleotide would explain its inability to engage a mechanism of kinetic partitioning and substrate remodelling, despite binding the J-domain. Likewise, the BiP^{V461F} mutation's effect on substrate binding also precludes this mechanism. BiP's ability to regulate P58's association with misfolded protein thus fits well with the latter's proposed role as an intermediary that assists protein folding in the ER lumen.

Materials and methods

Mammalian cell culture and *in vivo* protein expression

CHO.K1 and 293T cells were cultured in standard conditions and transfected with FuGENE 6 transfection reagent (Roche) or by calcium phosphate co-precipitation, respectively. An expression plasmid encoding N-terminally FLAG-tagged mouse P58 (27–504) was constructed in the pFLAG-CMV-1 vector (Sigma) and deletion derivatives and point mutations were introduced by PCR and confirmed by sequencing. The tagged protein-coding sequence was then transferred to the pBABE-puro plasmid and pseudotyped retrovirus produced in 293T cells. These were used to infect CHO.K1 cells and select puromycin-resistant clones that stably express FLAG–P58 (27–504) and its mutant derivatives. Mammalian expression plasmid encoding VSV-G^{ts045}–GFP (Nehls *et al*, 2000) was a gift of Jennifer Lippincott-Schwartz.

Antisera, immunoblotting and immunoprecipitation

Antisera to mouse P58 and GFP were described earlier (Oyadomari *et al*, 2006). α -FLAG M2 monoclonal antibody, α -FLAG M1 affinity gel and EZView Red α -FLAG M2 Affinity gel were purchased from Sigma. Immunoblots were developed using HRP-conjugated donkey α -mouse (Zymed) or IRDye 800CW-conjugated goat α -mouse on an Odyssey Infrared Imaging System and analysed by Odyssey software (v2.1) (LI-COR).

To recover proteins associated with P58, CHO.K1 cells stably expressing FLAG–P58 were transfected with VSV-G^{ts045}–GFP plasmid and 48 h later were labelled with ³⁵S-Translabel for 30 min–1 h in the presence of 10 μ M MG132 and 5 μ g/ml BFA (LC Laboratories) under permissive (32°C) or non-permissive (41°C) conditions followed by cold chase. Cell lysates were prepared in a Ca²⁺-containing buffer (0.3% Triton X-100, 150 mM NaCl, 20 mM Hepes pH 7.4, 10% glycerol, 10 mM CaCl₂, 1 mM DTT, 1 mM PMSF, 4 μ g/ml aprotinin and 2 μ g/ml pepstatin A) and radiolabelled proteins were immunopurified with α -FLAG M1 affinity gel overnight. The bound proteins were eluted from α -FLAG M1 in 10 mM EDTA, 20 mM Tris pH 7.5, 0.02% Tween-20 or 500 μ g/ml FLAG peptide, resolved by SDS–PAGE and detected by the Storm phosphorimager (Molecular Dynamics). To dissociate BiP, immunopurified complexes were exposed to K buffer (20 mM Hepes pH 7.0, 75 mM KCl, 5 mM MgCl₂ and 0.01% Tween-20) at room temperature for 30 min in the absence or presence of ATP (5 mM), washed extensively with K buffer, eluted and analysed as above.

Bacterial expression, protein purification and analysis

Construction of plasmids and procedures for protein expression and analysis of BiP's ATPase activity are described in the Supplementary data.

To assay BiP binding, 10 nmol of the GST–P58–J-domain (384–470) wild-type (GST–J^{WT}) or mutant (GST–J^{H422Q}) proteins, or GST, was immobilized on glutathione sepharose and 10 nmol of purified BiP was added to K buffer and allowed to interact in the presence of 5 mM ATP or ADP for 1 h at 4°C. After four washes with K buffer, the remaining proteins were revealed by SDS–PAGE stained with Coomassie.

Chaperone association with denatured RNase A

Generation of a denatured or native RNase A affinity matrix is described in the Supplementary data. Lysates from the aforementioned

mammalian cells, or P58 purified from bacteria or CHO cells, were allowed to interact with 10 µl of either the native or denatured RNase A matrix in a total volume of 500 µl by rotation at 30°C for 1 h. The matrices and associated proteins were washed five times with cold buffer K and the associating proteins were eluted with 2 × Laemmli buffer, separated by SDS–PAGE and revealed by Coomassie staining or immunoblotting.

To study chemical crosslinking of P58 to denatured RNase A, lysates from 293T cells expressing FLAG-tagged P58 were prepared in buffer B (0.1% Triton X-100, 20 mM Hepes pH 7.4, 75 mM KCl, 10% glycerol and 1 mM EDTA) and allowed to interact with denatured RNase A as described above. After two washes with buffer K to remove loosely associated proteins, the complexes were incubated at 37°C for 30 min in the presence or absence of 5 mM ATP (Sigma) to release endogenous associated BiP, followed by four more washes with buffer K. Remaining associated proteins were covalently crosslinked to the immobilized denatured RNase A with 50 µM dithiobis(succinimidylpropionate) (DSP) (Pierce) in a total volume of 500 µl at 4°C on rotation for 1 h followed by quenching with 100 mM Tris pH 7.5 for 15 min at 4°C. Non-covalently associated proteins were eluted with 4% SDS, 100 mM Tris pH 6.8 and the matrices were further washed with RIPA three more times. Proteins covalently crosslinked to the denatured RNase A matrix were then recovered by eluting with Laemmli buffer containing 100 mM DTT (which reduces an S–S bond that joins the crosslinker's arms) and analysed by immunoblotting.

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Release of P58 from denatured RNaseA

Lysates from CHO cells stably expressing FLAG-tagged P58 or purified bacterially expressed P58 and its mutants were allowed to interact with denatured RNase A matrix in buffer B as described above. After three washes with buffer K, the assembled complexes were challenged with 9 µM of purified bacterially expressed BiP in the presence of 5 mM ATP or ADP in a total volume of 400 µl of buffer K at 37°C for 30 min. Where indicated, bacterially expressed purified GST–P58–J-domain (384–470) (9 µM) was added. Dissociated proteins were removed by three washes with buffer K, the remaining material was eluted with Laemmli buffer and analysed by immunoblotting.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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