Regulated release of ERdj3 from unfolded proteins by BiP

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DnaJ proteins often bind to unfolded substrates and recruit their Hsp70 partners. This induces a conformational change in the Hsp70 that stabilizes its binding to substrate. By some unknown mechanism, the DnaJ protein is released. We examined the requirements for the release of ERdj3, a mammalian ER DnaJ, from substrates and found that BiP promoted the release of ERdj3 only in the presence of ATP. Mutations in ERdj3 or BiP that disrupted their interaction interrupted the release of ERdj3. BiP mutants that were defective in any step of the ATPase cycle were also unable to release ERdj3. These results demonstrate that a functional interaction between ERdj3 and BiP, including both a direct interaction and the ability to stimulate BiP’s ATPase activity are required to release ERdj3 from substrate and support a model where ERdj3 must recruit BiP and stimulate its high-affinity association with the substrate through activation of ATP hydrolysis to trigger its own release from substrates. On the basis of similarities among DnaJs and Hsp70s, this is likely to be applicable to other Hsp70–DnaJ pairs.

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Introduction

The Hsp70 family of molecular chaperones is a highly conserved, widely expressed, and well-studied group of proteins. These chaperones are found in all organisms where they have a function in every cellular organelle and are essential for nearly all cellular processes. The binding of Hsp70 proteins to non-native structures on a vast array of substrate proteins can serve to stabilize folding intermediates, prevent their aggregation, and aid in protein folding and assembly. This is achieved through direct interaction of the C-terminal substrate-binding domain (SBD) of Hsp70 proteins with exposed hydrophobic residues on substrate proteins. Peptide-binding studies suggested a preference for extended chains in which the hydrophobic amino acids would be oriented in a single direction to engage the peptide-binding pocket of the Hsp70 protein, a possibility that is supported by NMR (Landry et al, 1992) and crystallographic (Zhu et al, 1996) studies. The binding and release of substrates to the SBD of Hsp70 proteins are tightly regulated by the highly conserved N-terminal nucleotide-binding domain (NBD) (Liberek et al, 1991b), which can bind either ATP or ADP. When ATP occupies the cleft of the NBD, the SBD is in an open configuration, with both a high on and off rate for unfolded proteins. The hydrolysis of ATP to ADP results in a closure of the lid on the SBD, which stabilizes the interaction with bound proteins. Discharge of the unfolded protein occurs when ADP is released and exchanged for ATP. This reopens the lid on the SBD, which allows the bound substrate to be released and provides an opportunity for it to fold. A number of recent studies shed light on the interaction between the two domains, which controls the activity of this group of chaperones (Jiang et al, 2005; Vogel et al, 2006a, b; Liu and Hendrickson, 2007; Awad et al, 2008).

The Hsp70 ATPase cycle, which is essential to the chaperoning process, is controlled by a number of cofactors that regulate either ATP hydrolysis or nucleotide exchange. DnaJ was originally identified along with DnaK (Hsp70) in a genetic screen in Escherichia coli for genes that are required for DNA replication (Saito and Uchida, 1977; Yochem et al, 1978). Later it was shown that DnaK and DnaJ are in the same genetic pathway and that DnaJ stimulates the ATPase activity of DnaK, thereby stabilizing the binding of DnaK to substrates (Liberek et al, 1991a). As is the case with Hsp70s, DnaJ proteins are present in all organisms and all organelles, and the number of DnaJ proteins in an organism often exceeds the number of Hsp70 proteins present (Caplan et al, 1993; Cheetham and Caplan, 1998). All DnaJ proteins possess a highly conserved ~70 amino-acid ‘J’ domain, which contains an invariant tripeptide sequence, His-Pro-Asp, that is required to interact with the ATP-bound form Hsp70 proteins (Mayer et al, 1999). Similar to Hsp70 proteins, at least some DnaJ proteins can bind directly to unfolded substrates (Cheetham and Caplan, 1998; Fan et al, 2003). Peptide-binding studies for E. coli DnaJ revealed significant overlap with the peptides that bound DnaK (Rudiger et al, 2001), arguing that DnaJ was likely to also bind to extended hydrophobic residues on unfolded proteins. This possibility was supported by crystallographic data obtained for a peptide bound to the yeast cytosolic DnaJ protein, Ydj1 (Li et al, 2003). The fact that DnaJs specifically interact with the ATP-bound form of Hsp70 led to a model (Mayer et al, 1999) where DnaJ proteins would bind first to unfolded proteins, recruit the ATP-bound or ‘open’ form of Hsp70 to the substrate, and then stimulate its ATPase activity to ‘close’ it onto the substrate more stably. This model was supported by

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data showing that a cytosolic DnaJ protein bound to nascent chains extruding from the ribosome before the Hsp70 protein did (Hendrick et al., 1993), and by *in vitro* binding studies with DnaK, DnaJ, and denatured luciferase (Szabo et al., 1994) and with HscA and HscB, a specialized Hsp70-DnaJ pair of *E. coli* (Silberg et al., 2004). However, these studies did not reveal how DnaJ proteins were released from the substrate. Unlike Hsp70, DnaJ proteins do not bind to nucleotide and have not been demonstrated to exist in different conformational states.

The mammalian ER possesses at least six DnaJ family members (Brightman et al., 1995; Meyer et al., 2000; Tyedmers et al., 2000; Yu et al., 2000; Shen et al., 2002; Cunnea et al., 2003; Rutkowski et al., 2007; Petrova et al., 2008). One of these, ERdj3, was shown previously by our group to bind to a number of unfolded proteins in the ER that were BiP substrates (Shen and Hendershot, 2005). When the binding of wild-type and mutant (HPD→QPD) ERdj3 to several different substrates was compared, we consistently found that mutant ERdj3 bound quantitatively better and longer than wild-type ERdj3 (Shen and Hendershot, 2005). The present study was undertaken to better understand the requirements for releasing DnaJ proteins from substrates. Using a series of ERdj3 and BiP mutants, we found that release of ERdj3 was not simply due to a competition with BiP, but that a functional interaction between ERdj3 and BiP was required. We hypothesize that once ERdj3 has recruited BiP to the substrate it must stimulate BiP’s ATPase activity to induce high-affinity binding of BiP to the substrate, which produces the signal for ERdj3 release from substrate.

**Results**

**Comparison of the effects of J-domain mutations on ERdj3’s ability to associate with substrate both in vivo and in vitro**

It has been shown that the J domains of DnaJ proteins are important for interactions with their Hsp70 partners, where the signature HPD motif in the J domain has an indispensable function. We demonstrated earlier that a QPD mutation in ERdj3 abrogated its ability to interact with its ER Hsp70 partner, BiP, both physically and functionally (Shen and Hendershot, 2005). To determine whether the interaction with BiP was crucial for ERdj3’s ability to bind to unfolded substrates, we examined the ability of wild-type and mutant ERdj3 to bind to immunoglobulin heavy chain (γHC) both *in vivo* and *in vitro*. First, we co-expressed γHC along with HA-tagged versions of either wild-type ERdj3 or two different J-domain mutants (both QPD and ΔJ) in COS-1 cells. Co-immunoprecipitation experiments were performed on 3,3′-dithio-bis (propionic acid N-hydroxysuccinimide ester) (DSP)-crosslinked cell lysates. We found that J-domain mutations did not negatively affect the ability of the mutant ERdj3 proteins to bind to γHC *in vivo* (Figure 1A). In fact, in both cases there was actually more binding of the QPD and ΔJ mutants to γHC as compared with the binding of wild-type ERdj3 (compare lanes 3 and 7 to lanes 1 and 5). Wild-type and mutant ERdj3 proteins were also expressed in COS-1 cells alone. Protein A Sepharose beads did not precipitate any of the three proteins (Supplementary Figure S1), demonstrating that the binding observed in Figure 1A is dependent on the co-expression of γHC. These data suggested two things; first, as these mutants are unable to interact with BiP, ERdj3 might bind directly to unfolded substrates, and second these ERdj3 mutants might have a higher affinity for substrate or some component of the ER chaperone complex (Meunier et al., 2002). To directly test the first possibility, we developed an *in vitro* assay to examine the binding of ERdj3 to γHC in the absence of other resident ER chaperones and folding enzymes. It was based on our previous demonstration that BiP can be released from isolated γHC *in vitro* with ATP leaving the γHC in a conformation that allows them to reassociate with exogenously added BiP (Wei et al., 1995). As shown in Figure 1B (lane 1), ERdj3 can be isolated with γHC only when cells are pretreated with a membrane permeable crosslinker, DSP, whereas BiP’s association with γHC is detectable even without crosslinking (Figure 1B, lane 3). However, addition of ATP releases BiP from the γHC (Figure 1B, lane 2). These free γHC were used for binding to *in vitro* translated ERdj3 proteins (Figure 1C). We found that unlike the *in vivo* binding assays, the QPD mutant bound to γHC at similar levels as observed for wild-type ERdj3 (Figure 1C, lanes 4 and 5). These data revealed that ERdj3 associates directly with substrates and also argues that the enhanced binding of ERdj3 mutants to γHC *in vitro* is unlikely to be due to their having a higher affinity for substrate. Instead, it suggested that something else in the cell might be contributing to the difference between wild-type and mutant ERdj3’s association with substrate. On the basis of our previous data showing that mutant ERdj3 remains bound to unfolded Ig light chains much longer than wild-type ERdj3 (Shen and Hendershot, 2005), we hypothesized that the J-domain mutations might be affecting release of the ER DnaJ proteins from substrates and that release might be dependent on a functional interaction with BiP.

**Development of an in vitro system to detect binding and release of ERdj3 from substrates**

Because the isolation of free γHC was somewhat cumbersome, we wished to develop a simpler *in vitro* binding assay. We chose denatured firefly luciferase (D-Luc), because it has been widely used as an *in vitro* substrate for other DnaJ family members, including *E. coli* DnaJ and two yeast cytosolic DnaJ family members Sis1 and Ydj1 (Szabo et al., 1996; Schumacher et al., 1996; Lu and Cyr, 1998a,b). To determine whether denatured luciferase could serve as an ERdj3 substrate *in vitro*, we first examined the ability of wild-type ERdj3 to bind to either native or heat-denatured luciferase in solution. For these experiments, luciferase was denatured with heat instead of urea, because we did not want to interfere with the protein–protein interactions required for association and immunoprecipitation. ERdj3 was allowed to interact with native or denatured luciferase and the samples were immunoprecipitated with either a polyclonal anti-ERdj3 antisera or Protein A Sepharose beads. The association of luciferase was determined by immunoblotting with an anti-luciferase antibody. We found that indeed the binding of ERdj3 to denatured luciferase (D) was readily detectable, whereas its binding to native luciferase (N) was below the level of detection (Figure 2A). This distinction in binding is in keeping with ERdj3 acting as a chaperone and demonstrated that denatured luciferase could be used as an *in vitro* substrate for ERdj3.

Next a modified ELISA was developed, which would allow us to readily examine the ability of ERdj3 to bind to luciferase...
under multiple conditions. For this assay, the luciferase was chemically denatured, as has been done in a number of other studies (Szabo et al., 1994; Lu and Cyr, 1998a, b). First, we tested the binding of wild-type ERdj3 and the QPD mutant to chemically denatured luciferase, which was bound to 96-well plates. Similar to the \textit{in vitro} binding of these proteins to γHC, we found that both wild-type ERdj3 and the QPD mutant associated equally with chemically denatured luciferase (Figure 2B). Thus, mutation of HPD sequence to QPD did not affect the binding of ERdj3 to chemically denatured luciferase.

**ATP does not affect the binding of wild-type ERdj3 or the QPD and HPN mutants to substrate in vitro**

Before testing our hypothesis that ERdj3 release from substrates occurs in response to a functional interaction with BiP, it was necessary to set up an assay to saturate the binding of ERdj3 to luciferase and to ensure that incubation with ATP did not affect this binding. Increasing concentrations of wild-type (Figure 3A), QPD (Figure 3B), or HPN (Figure 3C) recombinant ERdj3 proteins were added to luciferase-coated wells in the absence (Figure 3, solid bar) or presence (Figure 3, hatched bar) of ATP. We found that all three proteins reached saturation binding at concentrations of ~500 nM and that the inclusion of ATP in the binding buffer did not affect ERdj3’s ability to bind to substrate. Thus, in the following experiments 500 nM ATP was used. Quantitation of the amount of luciferase and ERdj3 that were bound to the wells revealed the ERdj3 bound to denatured luciferase at a little under a 1:1 ratio (Supplementary Figure S2).

**BiP promoted the release of wild-type ERdj3 from chemically denatured luciferase in the presence of ATP**

If a functional interaction between BiP and ERdj3 is critical for releasing ERdj3 from substrates, the amount of ERdj3 that

![Figure 1 ERdj3 binds to γHC directly.](image1)

![Figure 2 WT and QPD ERdj3 bind to denatured luciferase similarly \textit{in vitro}](image2)
is associated with substrate is expected to decrease in the presence of BiP in an ATP-dependent manner. To examine this possibility, increasing amounts of BiP were added to wells containing ERdj3 bound to denatured luciferase in the absence and presence of ATP (Figure 4A). We found that the addition of increasing amounts of BiP in the absence of ATP did not affect ERdj3’s association with denatured luciferase (Figure 4A, solid bar), although there was a detectable increase in the binding of BiP to the substrate (Figure 4B, solid bar), which demonstrates that release of ERdj3 does not occur through a simple competition between these two chaperones for substrate and further suggests that BiP- and ERdj3-binding sites are not completely overlapping. However, when ATP was included with BiP, we found that the ability of BiP to release ERdj3 was dependent not only on the concentration of BiP but also required ATP (Figure 4A, hatched bar). The binding of BiP to denatured luciferase did not increase (Figure 4B, hatched bar) when ERdj3 was released (Figure 4A, hatched bar), again suggesting that ERdj3 release does not occur due to a simple competition with BiP. To examine this from the other direction, we first bound either wild-type BiP or a BiP mutant that cannot interact with ER DnaJ proteins (R197H) (Awad et al., 2008) to denatured luciferase and measured the ability of ERdj3 to release them. We found that the addition of ERdj3 did not induce a reduction in the binding of either wild-type or mutant BiP to luciferase-coated well (Figure 4C, solid and hatched bars), even though we could readily measure the binding of ERdj3 to luciferase (Figure 4C, checkered bars). This further argues that the release of ERdj3 does not occur due to a simple competition between BiP and ERdj3 for binding sites on the substrate.

### BiP mutants that do not interact with ERdj3 failed to promote the release of ERdj3 from substrate

The requirement of ATP for BiP to release ERdj3 from the substrate suggested that a functional interaction between BiP and ERdj3 might be necessary. To test this possibility, we examined the ability of a number of different BiP mutants to release ERdj3 from luciferase. A highly conserved arginine on
the ATPase domain of Hsp70 proteins (R197 in BiP) has been shown to be essential for interaction with the HPD motif on DnaJ proteins (Gassler et al., 1998; Suh et al., 1998; Alder et al., 2005). We recently made three substitutions at this site (R197H, R197A, and R197E), all of which have ATPase activity equal to or greater than wild-type BiP, but none of them can bind or be further stimulated by J proteins (Awad et al., 2008). Two other BiP mutants were also tested; a G227D mutant that cannot bind to ATP and a T37G mutant that cannot undergo the ATP-induced conformational change that is required for its chaperoning activity (Wei et al., 1995). Recombinant proteins corresponding to each of these mutants were made and tested both for their ability to bind to luciferase and to release wild-type ERdj3. We found that all five BiP mutants were able to bind equivalently to luciferase when tested at a concentration of 5 μM (Figure 5A), whereas only very low levels of background binding to the wells were observed for all of these proteins when luciferase was not present (Supplementary Figure S3). Although all of the mutants bound to luciferase both alone and in the presence of ERdj3 (Figure 5C), none of them was able to release ERdj3 from this substrate even in the presence of ATP (Figure 5B). As only wild-type BiP was able to release ERdj3 from substrate, it was possible that the loss of ERdj3 was due to refolding of luciferase on the plate. To exclude this possibility, we examined the enzymatic activity of luciferase after interaction with ERdj3 and various BiP mutants and found no evidence of productive refolding, even though our assay could have detected as little as 0.05% renaturation (Supplementary Figure S4).

**Wild-type BiP does not release two ERdj3 mutants, QPD and HPN, from luciferase**

To further explore the possibility that a functional interaction between ERdj3 and BiP was required for ERdj3 release, we produced two ERdj3 proteins in which the HPD motif had been mutated and therefore should not interact functionally with wild-type BiP based on previously defined DnaJ mutants (Wall et al., 1994). The first of these, HPD→QPD, disrupts binding to BiP and stimulation of its ATPase activity without interfering with the ability of this mutant to bind to substrates (Shen and Hendershot, 2005). The second mutant HPD→HPN corresponds to a DnaJ mutant that was defective in interacting with wild-type DnaK (Suh et al., 1998). We first tested the ability of these two mutants to bind to BiP in vitro and to stimulate its ATPase activity. Wild-type recombinant BiP was immobilized on Protein A Sepharose beads by immunoprecipitation and wild-type and mutant ERdj3 proteins were allowed to bind in the presence of ATP (Figure 6A). We found that both mutations interfered with the ability of ERdj3 to bind to BiP, which is in keeping with data from a number of other DnaJ family members (Tsai and Douglas, 1996; Kelley and Georgopoulos, 1997; Wittung-Stafshede et al., 2003). The ability of the mutant ERdj3 proteins to stimulate the ATPase activity of BiP was compared with that of wild-type ERdj3. We found that wild-type ERdj3 stimulated BiP’s ATPase activity about two-fold, which is in keeping with previous data obtained with only the J domain and glycine/phenylalanine regions of ERdj3 (Shen and Hendershot, 2005), whereas the HPN and QPD mutants were unable to appreciably increase the hydrolysis of ATP (Figure 6B).

We next performed an experiment similar to those described in Figures 4 and 5, except that this time we were asking whether wild-type BiP was only capable of releasing wild-type ERdj3 or whether it was also able to release the two ERdj3 mutants that did not functionally interact with BiP. If BiP released the ERdj3 mutants, it would argue that a functional BiP–ATP–substrate interaction was required, but that there was no need for a functional BiP–ERdj3 interaction. We found that only wild-type ERdj3 was released by wild-type BiP in the presence of ATP, whereas both the QPD and HPN mutants remained bound to the substrate even in the presence of ATP (Figure 6C). The combination of this experiment and the previous one (Figure 5A) demonstrates that both a BiP–ATP–substrate interaction and a functional BiP–ERdj3 interaction are required to release ERdj3 from substrate. In a search for allele-specific suppressors of DnaJ HPN and QPD mutants, no suppressors were found for the QPD mutant, but three different DnaK mutants were identified that restored growth at temperatures that were non-permissive for the HPN mutant (Suh et al., 1998). Of these, the DnaK R167H mutant (analogous to our R197H mutant) bound better than wild-type DnaK to the DnaJ HPN mutant. Thus, we wished to determine whether our HPN ERdj3 mutant and our R197H BiP mutant would constitute a functional pair that could rescue the inability of HPN ERdj3 to be released from substrate. However, we found that the HPN mutant was as defective in binding to R197H BiP mutant as either wild-type ERdj3 or the QPD mutant and was unable to significantly

![Figure 5](image-url)
trigger the release of the HPN mutant (Supplementary Figure S5). Thus, unfortunately the HPN ERdj3 mutant did not appear to re-establish a functional pair with the R197H BiP mutant by this criterion.

**BiP and ATP must be added simultaneously to the ERdj3–substrate complex to promote ERdj3 release**

To determine whether recruitment of BiP to the site of ERdj3–substrate interaction was sufficient to release ERdj3 or if it was important that BiP also hydrolyse ATP, we included a BiP mutant (E201G) that was able to interact with DnaJ proteins (Petrova et al., 2008) but that was defective in ATP hydrolysis (Gaut and Hendershot, 1993). We found that even though ERdj3 would be expected to recruit the E201G mutant locally, this BiP mutant was as unable as the R197H BiP mutant, which could not interact with ERdj3 to induce the release of ERdj3 under any condition (Figure 7A and B).

We also tested whether ATP needed to be included simultaneously to trigger release. When wild-type BiP was allowed to bind to the ERdj3–substrate complex in the absence of ATP and then washed to remove unbound BiP, challenging this complex with ATP did not lead to the release of ERdj3 (Figure 7A), even though it did induce the release of BiP (Figure 7C). Taken together, these data argue that BiP must be recruited locally to the site of ERdj3–substrate interaction and that ERdj3 must induce ATP hydrolysis in BiP to receive a signal to be released.

**Discussion**

There is now a significant amount of data to argue that DnaJ proteins bind to unfolded proteins initially and due to their ability to interact specifically with the ATP form of Hsp70s serve to recruit the open form of the Hsp70 to the substrate. Hsp70 proteins must be able to interact with a DnaJ protein as well as to bind and hydrolyse ATP to be efficiently recruited to the substrate (Wawrzynow et al., 1995a). Much less is understood about how DnaJ proteins leave the substrate once Hsp70 has been recruited, although several models have been proposed. First, it is possible that once the DnaJ protein contacts an Hsp70, it releases the unfolded protein and the Hsp70 captures it (Rudiger et al., 2001). The identification of stable DnaJ–Hsp70–substrate complexes (Szabo et al., 1994; Wawrzynow et al., 1995a; Han and Christen, 2003; Shen and Hendershot, 2005) makes this scenario less likely, as do our data showing that the E201G BiP mutant, which does make contact with ERdj3, is unable to induce the release of ERdj3. Second, it is possible that, in addition to the conformational change that DnaJ induces in the Hsp70 protein, upon substrate binding and ATP hydrolysis the Hsp70 protein provides a reciprocal signal to the DnaJ protein that causes its release. It is known that type I and II DnaJ proteins have a second interaction site with the SBD of Hsp70s (Laufen et al., 1999), but it is not clear which domain of DnaJ is involved in this binding (Wall et al., 1995; Wawrzynow and Zylicz, 1995b; Linke et al., 2003; Sahi and Craig, 2007), but if it involved the SBD of DnaJ proteins, this second interaction could induce its release. The fact that similar requirements for release were observed with P58/DNAJ3c, a type III DnaJ protein (Petrova et al., 2008), makes this possibility less likely as the SBD of this group of proteins does not evidently resemble that of type I and II DnaJ proteins and probably does not share the same orientation to the J domain (Gruschus et al., 2004). However, in the absence of a full-length structure for the various types of DnaJ proteins, we do not know the relative orientation of J domains to SBD.

Finally, it has been proposed that once the J domain interacts with the NBD of Hsp70, it reorients the Hsp70 and in some way wrenches the substrate from DnaJ (Landry, 2003). Our demonstration that wild-type BiP, which binds the substrate but cannot release the QPD or HPN mutant, is compatible with this model, as the absence of the BiP–ERdj3 interaction would not allow BiP to wrench the substrate from ERdj3. This wrenching would need to happen before ATP is
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Hydrolysed, as DnaJ domains lose affinity for the ADP-bound form of Hsp70 (Wawrzynow and Zylicz, 1995b). Our finding that ATP must be added simultaneously for the BiP-mediated release to occur argues that the formation of this three-way complex is critical to release, as in the absence of ATP, BiP would not interact with ERdj3 and be concentrated locally. However, the fact that E201G BiP is able to interact with substrate and ERdj3 but cannot induce the release of ERdj3 argues that, although necessary, the formation of the three-way complex is not sufficient to wrench the DnaJ protein from the substrate. Instead, our data argue that in addition to recruiting BiP to the substrate, the DnaJ protein may be able to ‘sense’ that it has stimulated the ATPase activity of BiP or that BiP must assume the capability to wrench the substrate from the DnaJ partner in an ATP-dependent manner.

Our discovery that the release of ERdj3 from substrate is dependent on a functional interaction with BiP is likely to be true of other DnaJ–Hsp70 pairs. Recent data from Petrova et al demonstrate a similar requirement for the release of another ER-localized DnaJ protein, P58, from substrate. Mutations in either P58 or BiP that disrupted the interaction between them blocked the release of P58 from misfolded RNase A. In addition, an earlier in vitro study found that Ydj1 bound to a substrate and prevented its aggregation, but it could not fold the protein unless Hsp70 and ATP were present (Lu and Cyr, 1998a, b; Fan et al, 2005). In view of our data, it is reasonable to suggest that DnaJ remained bound to the substrate in the absence of DnaK, thereby preventing its aggregation, but also preventing it from folding. Only in the presence of ATP would both chaperones be released allowing the substrate to fold. We would speculate, based on our data, that in the absence of ATP that both DnaK and DnaJ might bind, but folding would not occur.

Our previous characterization of the interaction of ERdj3 with substrates in cultured cell lines revealed that wild-type ERdj3 disappeared from BiP–substrate complexes long before folding was complete, whereas a QPD mutant remained associated with the substrate (Shen and Hendershot, 2005). This led us to speculate that the prolonged binding of the QPD mutant to substrates could be due to its inability to recognize that BiP had bound productively to the substrate. The data presented here confirm that the release of ERdj3 from substrate, at least in vitro, required an interaction between the J domain of ERdj3 and NBD of BiP and argue that indeed this is the reason for prolonged association of mutant ERdj3 with substrates in vivo. The in vitro release of ERdj3 from substrate required BiP’s ATPase activity (T37G, G227D, and E201G), its ability to interact with ERdj3 (R197H), and presumably its substrate-binding ability (Petrova et al, 2008). These requirements would ensure that once a DnaJ protein engages an unfolded substrate, it would remain bound to prevent aggregation until it had recruited an open form of Hsp70 to the substrate, allowed the Hsp70 to initially associate with the substrate and then hydrolyse ATP to form a more stable interaction. Only when all of these steps had occurred would the DnaJ protein release from the Hsp70 and from the substrate. This scenario is consistent with most published data showing that DnaJ proteins bind first and more transiently than Hsp70, which remain associated until folding is complete.

In summary, our studies reveal that BiP promotes the release of ERdj3 from substrates in the presence of ATP. This is not due to a competition for binding sites on the substrate once BiP is recruited through its association with ERdj3, but rather it requires a functional interaction between ERdj3 and BiP. This includes both the ability to physically interact with each other and the ability of ERdj3 to stimulate the ATPase activity of BiP. The fact that similar data were observed with another ERdj3–BiP pair (Petrova et al, 2008) argues that this mechanism is likely to be used for the release of other DnaJ proteins from substrates.

Materials and methods

Cell culture, transfection, and immunoprecipitation

COS-1 monkey kidney fibroblast cells were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and 1% Fungisone in 3% CO₂. Cells were transfected with the indicated vectors using the FuGene 6 transfection reagent (Roche Diagnostics), and after 48 h post-transfection, cells were labelled with [35S]Translabel (Amersham Biosciences) for 3 h. Cells were treated with 150 µg/ml DSP, a membrane-permeable crosslinking reagent (Sigma-Aldrich) for 1 h on ice. Cell lysates were prepared using an NP-40 lysis buffer and immunoprecipitated with indicated antibodies followed by binding to Protein A Sepharose beads. The immunoprecipitated complexes were analysed by SDS-PAGE under reducing conditions, and the signal was detected using Amplify (Amersham Biosciences) for radiographic visualization.
In vitro translation and heavy chain-binding assay

Ag8.8 murine plasmacytoma cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and 1% Fungizone in 5% CO2. Cells were metabolically labelled for 16 h with [35S]Translabel (Amersham Biosciences) and immunoprecipitated with either anti-ERdj3 polyclonal anti-serum followed by Protein A as described earlier (Wei et al., 1995). To release BiP from heavy chains, non-crosslinked samples were supplemented with 1 mM MgCl2, 25 mM KCl, and 1 mM ATP. These samples are the source of free yHC used in the in vitro binding assays, except that the beads were washed an additional three times in PBS to reduce detergent in the samples, which interferes with the ability of ERdj3 to remain associated with unfolded substrates (Shen and Hendershot, 2005).

The cDNAs encoding wild-type and mutant (H35Q) ERdj3 were transcribed from the T7 promoter of 3HADSL-ERdj3 (Stratagene) and translated using [35S]methionine (Amersham Biosciences) and the TNT-coupled rabbit reticulocyte lysate (Promega). Equivalent counts for the two protein products were loaded directly on reducing SDS-polyacrylamide gels or incubated with either uncoupled Protein A Sepharose beads (washed three times in PBS), or with Protein A Sepharose beads to which free yHC were bound. After incubating for 1 h on ice, the beads were washed three times with PBS and bound proteins were subjected to reducing SDS-PAGE.

IP western

Firefly luciferase (Promega) was left untreated (N) or heat denatured (D) at 42°C for 1 h. In both cases, 0.5 μg of protein was incubated with recombinant wild-type ERdj3 protein (2.0 μg) in PBS and immunoprecipitated with either anti-ERdj3 polyclonal anti-serum followed by Protein A Sepharose beads or with Protein A Sepharose beads alone. Bound proteins were subjected to reducing SDS-PAGE and then transferred to a PVDF membrane (Bio-Rad), which was blotted with an anti-luciferase antisemum (1:1000) (Promega). Donkey anti-goat IgG conjugated to HRP (1:5000) was used as a secondary antibody and the signal was detected by chemiluminescence (ECL).

Protein purification

Expression of His-tagged wild-type and mutant ERdj3 was induced in E. coli M15 cells with 0.1 mM isopropyl β-D-thiogalactoside (Sigma-Aldrich) followed by growth for 18 h at 18°C. The recombinant proteins were purified on Ni2+-agarose columns under non-denaturing conditions (Qiagen QIAexpress system), dialysed in 25 mM sodium phosphate buffer (pH 7.0), containing 150 mM NaCl, 0.02% Triton X-100, 50% glycerol, and a protease inhibitor cocktail (Roche), and stored at −80°C. His-tagged wild-type and mutant BiP proteins were induced with 1 mM IPTG for 2 h at 37°C, purified on Ni2+-agarose columns, dialysed and stored in 20 mM HEPES buffer (pH 7.2) with 50 mM KCl, 5 mM MgCl2, 0.1% NP-40, 50% glycerol, and a protease inhibitor cocktail.

Measurement of complex formation between ERdj3 proteins and denatured luciferase

Firefly luciferase was denatured in a buffer containing 7 M urea, 25 mM HEPES (pH 7.5), 50 mM KCl, 5 mM EDTA, 5 mM MgCl2, and 5 mM dithiothreitol at room temperature for 40 min and then diluted into PBS containing 0.05% BSA (final concentration 5 μg/ml). A portion of 100 μl of this solution was added into each well of a 96-well microtitre plate purchased from Thermo (Immulon 2HB Wells). Wells were washed with PBS and blocked with 200 μl PBS containing 1% BSA for 1 h at room temperature. The indicated concentrations of wild-type or mutant ERdj3 in 100 μl of PBS with 0.05% BSA was added to the wells and incubated for 1 h at room temperature, followed by washing with PBS to remove unbound ERdj3. The amount of ERdj3 that remained bound to denatured luciferase was detected with a polyclonal anti-ERdj3 antisemum (Shen and Hendershot, 2005) followed by donkey anti-rabbit IgG conjugated to alkaline phosphatase (Promega), 4-Nitrophenyl phosphate disodium salt hexahydrate (Sigma-Aldrich) was added, and after stopping the reaction with 0.75 M NaCl, the plates were read on a spectrophotometer at a wavelength of 405 nm. Negative controls were set up for each plate in wells that did not contain either denatured luciferase, ERdj3, or each of the antibodies, but which included all the other steps of the reaction.

Release of ERdj3 from luciferase

To detect the amount of BiP binding to denatured luciferase, recombinant hamster BiP was added to wells coated with denatured luciferase instead of ERdj3, and incubated as above, except that a rabbit polyclonal anti-rodent BiP antisemum was used to detect BiP binding. To test the ability of wild-type and mutant BiP to release ERdj3 from luciferase, ERdj3 was first bound to luciferase as above. After washing away unbound ERdj3, the indicated amounts of recombinant BiP proteins were added to the wells in PBS containing 1 mM MgCl2, 25 mM KCl, and either 1 mM ATP or no nucleotide. The plates were incubated for 1 h at room temperature, and the amount of ERdj3 or BiP associated with the denatured luciferase was determined as above.

Luciferase activity assay

Triplicate samples of serial dilutions of native (N) or denatured (D) luciferase in PBS were added to a 96-well microtitre plate. Next, 100 μl of luciferase assay reagent (Promega) was injected into each well and chemiluminescence was read immediately on a 1420 Multilabel Counter (PerkinElmer). To test whether denatured luciferase was able to be refolded in the wells, 96 wells were coated with 0.5 μg D-Luc and reactions were performed as described above. The denatured luciferase was further incubated alone, with ERdj3, or with ERdj3 and BiP in the presence or absence of ATP. The luciferase assay reagent was injected into each well, and luciferase activity was measured on the plates. Each experimental condition was performed in triplicate.

ATPase assay

ATPase assays were performed as described earlier (Chevalier et al., 1998). Briefly, 1 μM of the various recombinant BiP proteins was incubated alone or with 0.5 μM of the indicated full-length ERdj3 proteins at 37°C for 20 min in ATPase buffer containing [γ-32P]ATP (PerkinElmer). After chromatography, the radioactive ATP and free phosphate signals were quantified by phosphomager analysis (Molecular Dynamics, Sunnyvale, CA) using Image Quant software. The free phosphate signal was expressed as a percentage of the total phosphate signal. Data were deduced from three independent experiments, and the error bars represent standard deviations (s.d.).

Supplementary data

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

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