Dissection of Structural and Functional Requirements That Underlie the Interaction of ERdj3 Protein with Substrates in the Endoplasmic Reticulum*

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Background: ERdj3 is a dimeric type I DnaJ co-chaperone for BiP, the ER Hsp70.

Results: Mutational analyses revealed the requirements for ERdj3 substrate binding and release in cells.

Conclusion: ERdj3 does not rebind substrates after release, which is linked to substrate half-life.

Significance: This study provides mechanistic insights into how ERdj3 aids BiP in deciding the fate of nascent ER proteins.

ERdj3, a mammalian endoplasmic reticulum (ER) Hsp40/DnaJ family member, binds unfolded proteins, transfers them to BiP, and concomitantly stimulates BiP ATPase activity. However, the requirements for ERdj3 binding to and release from substrates in cells are not well understood. We found that ERdj3 homodimers that cannot stimulate the ATPase activity of BiP (QPD mutants) bound to unfolded ER proteins under steady state conditions in much greater amounts than wild-type ERdj3. This was due to reduced release from these substrates as opposed to enhanced binding, although in both cases dimerization was strictly required for substrate binding. Conversely, heterodimers consisting of one wild-type and one mutant ERdj3 subunit bound substrates at levels comparable with wild-type ERdj3 homodimers, demonstrating that release requires only one protomer to be functional in stimulating BiP ATPase activity.

Co-expressing wild-type ERdj3 and a QPD mutant, which each exclusively formed homodimers, revealed that the release rate of wild-type ERdj3 varied according to the relative half-lives of substrates, suggesting that ERdj3 release is an important step in degradation of unfolded client proteins in the ER. Furthermore, pulse-chase experiments revealed that the binding of QPD mutant homodimers remained constant as opposed to increasing, suggesting that ERdj3 does not normally undergo reiterative binding cycles with substrates.

Hsp70 proteins are ubiquitously expressed molecular chaperones with highly conserved family members present in all organelles of eukaryotic organisms (reviewed in Ref. 1). The binding of unfolded proteins to the C-terminal substrate-binding domain of Hsp70s is regulated by the adenosine nucleotide bound to the N-terminal nucleotide-binding domain (reviewed in Ref. 2). The founding member of the Hsp70 family is Escherichia coli DnaK, which has provided many of the original insights into the nucleotide-regulated substrate binding cycle of this chaperone family (3–5). Critical steps in the cycle have subsequently been confirmed and extended for eukaryotic family members of various organelles (6–8). Hsp70 proteins cycle between an ATP-bound form, in which the substrate-binding domain is open and binds peptides and proteins with low affinity, and an ADP state, in which the lid of the substrate-binding domain closes over the bound substrate, thereby stabilizing the chaperone-substrate complex. Central to this binding cycle are DnaJ/Hsp40 family members, which can bind directly to peptides, nascent polypeptide chains, and unfolded substrates and deliver them to the ATP-bound form of Hsp70 proteins, while at the same time stimulating their low intrinsic ATPase activity to stabilize the binding of Hsp70 to the transferred substrate (reviewed in Ref. 9). Eukaryotic DnaJ family members are even more abundant than Hsp70s, and each organelar Hsp70 usually has multiple DnaJ proteins with which it can interact, arguing for important regulatory roles within the generic Hsp70 cycle. Unlike the Hsp70 proteins, the DnaJ family members are much less well conserved (reviewed in Refs. 9 and 10). Type I family members possess all the domains found in E. coli DnaJ. This includes an N-terminal J domain that contains the signature HPD peptide sequence, which is crucial for J protein-induced acceleration of Hsp70 ATPase activity (11–13), followed by a G/F-rich flexible region, domain I, which contains a substrate interaction site (14–16), a cysteine-rich domain II that contributes to substrate binding via a poorly understood mechanism (17, 18), and a C-terminal domain III that promotes formation of homodimers, which is also critical for substrate interaction. Type II family members lack only the cysteine-rich domain, whereas type III DnaJ proteins contain only the ~90 amino acid J domain flanked by other unrelated domains that contribute to functional specificity of the individual proteins (10, 19).
The mammalian ER\textsuperscript{5} has a single conventional Hsp70 protein, BiP, and at least seven DnaJ family proteins that have luminal J domains (20). Erdj3 is the only ER family member that possesses all the domains found in type I DnaJ proteins (16). Like DnaJ and other type I family members, it directly binds denatured proteins \textit{in vitro} (21) and was originally identified due to its interaction with Shiga toxin (22) and with unassembled immunoglobulin heavy chains (23). The substrate-binding domain of Erdj3 could be readily modeled (16) using the crystal structures of the substrate-binding domains of yeast cytosolic Ydj1p (15), which is very similar to that of \textit{Escherichia coli} DnaJ (17, 24), and the less well conserved yeast Sis1p (14). Their structures suggest that these DnaJ proteins form a dimeric, tong-like structure, which is required for them to grasp and chaperone unfolded substrates (25, 26). In the case of Ydj1, a phenylalanine residue in domain III near the C terminus was found to be critical for dimerization (27). Mutation of the corresponding phenylalanine in Erdj3 inhibited dimer formation and reduced its binding to denatured luciferase \textit{in vitro} (16).

Substrate-loaded DnaJ proteins interact with the ATP-bound or open form of their partner Hsp70 protein through their signature J domain (reviewed in Refs. 9 and 10). This three-way complex allows the transfer of the substrate to an Hsp70 and subsequent release of the DnaJ-like protein. However, the underlying mechanisms of transfer and release are not well understood and must account for the fact that the type I DnaJ proteins bind substrates as dimers and transfer them to an Hsp70 that binds unfolded proteins as a monomer. Using Erdj3 as a representative of the type I DnaJ family, we designed studies to better understand the mechanism of Erdj3 release from unfolded proteins in cells and to determine whether Erdj3 undergoes reiterative cycles of binding to these substrates. Erdj3 mutants that did not functionally interact with BiP initially bound to substrates similar to wild-type Erdj3 but were defective in substrate release. We found that BiP only needs to functionally interact with one arm of the Erdj3 dimer to release it from the substrate. We engineered Erdj3 mutants that were defective in substrate release so that they would form homodimers but would not dimerize with wild-type Erdj3 protein. Data obtained from co-expressing these mutants with wild-type Erdj3 argued against reiterative cycles of Erdj3 binding to substrates. Of note, in the case of two incompletely folded ER proteins, we found that release of wild-type Erdj3 was slower with a long-lived substrate when compared with a substrate that turned over more rapidly.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs and Mutant Design**—Plasmids with cDNAs encoding the NS-1 \( \kappa \) LC (28), \( \gamma \) HC, and BiP (29) have been previously described, as have the HA-tagged and untagged version of wild-type Erdj3, QPD (30), and F326D (16) mutants. Plasmids encoding for untagged Erdj3 or QPD (pSG5-Erdj3) were mutated by restriction-free cloning (31) to create Erdj3 and QPD F326C or F326C \( \Delta \gamma \), in which amino acids 329–358 are deleted. The primers used for mutation of Phe-326 to C were ordered from Life Technologies: forward, 5’-GATAATC-ACCTTTTGATGGATTGCTCAAAAAGAAGTTAAGG-3’ and reverse, 5’-CTCTGTTAATCTGTTCCTTTTG-GACAATCCACATCAAATGTATTATC-3’. The primers used for deletion of amino acids 329–358 were: forward, 5’-CACCCTTTGTTGATGTGGATTGTCCAAAAAGAAGCTTTA-ACAGGAAAGCCG-3’ and reverse, 5’-GGCCATTCTTCTGTTA-ACCTGTTCCTTTGGACATCCTACATCAAATGTG-3’. All mutations were confirmed by sequencing.

**Cell Culture and Transfection**—COS-1 and 293T cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and 100 units/ml penicillin-streptomycin solution in 3% and 5% CO\(_2\), respectively, at 37°C. Cells were transfected with the plasmids detailed in the figure legends using GeneCellin transfection reagent (BioCellChallenge) according to the manufacturer’s protocol.

**Preparation of Cell Lysates, Cross-linking, and Immunoprecipitation**—Cells were lysed 24 h after transfection in Nonidet P-40 lysis buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40 substitute, 0.5% deoxycholate) supplemented with 1 mM PMSF and protease inhibitors (Roche Applied Science). For cross-linking experiments, cells were washed and incubated on ice with 1 ml of cross-linking buffer (25 mM HEPES-KOH, pH 8.3 and 125 mM KCl) containing 150 \( \mu \)g/ml 3,3-dithio-bis (propionnic acid N-hydroxysuccinimide ester) (DSP) as described (23). The cross-linking reaction was quenched with 100 mM glycine before cell lysis. Immunoprecipitation was performed on clarified cell lysates with the indicated antibodies overnight at 4°C. Immune complexes were isolated with protein A-agarose beads, washed with Nonidet P-40 washing buffer (50 mM Tris/HCl, pH 7.5, 400 mM NaCl, 0.5% Nonidet P-40 substitute, 0.5% deoxycholate), and eluted in 2× reducing Laemmli buffer. Whole cell lysates were mixed with 4× reducing Laemmli buffer and analyzed by SDS-PAGE and followed by immunoblotting with the indicated antisera. For nonreducing SDS-PAGE, samples were lysed in Nonidet P-40 buffer as above with the addition of 10 mM \( \beta \)-mercaptoethanol.

**Metabolic Labeling and Pulse-Chase**—For metabolic labeling, cells were incubated in methionine- and cysteine-free DMEM labeling medium containing 10% dialyzed FBS for 30 min and labeled with 100 \( \mu \)Ci TRANS\( ^{35} \)S-Label (BM Biomedicals, PerkinElmer) for the indicated times. For pulse-chase experiments, the chase was initiated by removing the labeling medium, washing the cells twice with cold PBS, and adding DMEM containing 2 mM excess unlabeled methionine and cysteine followed by incubation at 37°C for the indicated times. To prevent protein synthesis during the chase, 5 \( \mu \)g/ml cycloheximide (Sigma) was included in the chase medium. At the indicated time points, cells were cross-linked and lysed with Nonidet P-40 lysis buffer. Cell lysates were immunoprecipitated as specified and analyzed by SDS-PAGE followed by autoradiography after incubating the gel in Amplify reagent (Amersham Biosciences).

**Western Blot and Antibodies**—Precipitated immune complexes or whole cell lysates were separated on SDS-polyacryl-
amid gels and transferred to Immun-Blot PVDF membranes (Bio-Rad). Proteins were detected with the indicated antibodies, visualized by incubating membranes with HRP-conjugated species-specific secondary antibodies (Santa Cruz Biotechnology), and developed with ECL reagent (Thermo Scientific) according to the manufacturer’s instructions. The polyclonal anti-ERdj3 and anti-BiP antisera were generated in our laboratory and described previously (30). The hybridoma cell line producing the mononclonal anti-HA antibody was a kind gift from Dr. Al Reynolds. The goat anti-human IgG heavy chain and goat anti-mouse κ light chain antibodies were purchased from Southern Biotech and the Hsc70 antibody from Santa Cruz Biotechnology.

Structural Modeling and Secondary Structure Prediction—A homology model of ERdj3 was built with YASARA Structure based on the available Thermus thermophilus DnaJ crystal structure (Protein Data Bank code 4J80) using the homology model builder macro implemented in YASARA Structure.

RESULTS

Steady State Binding of a QPD ERdj3 Mutant Is Reduced upon Co-expression of Wild-type ERdj3—The signature HPD sequence found in the J domain of all Hsp40 family members plays an important role in their functional interaction with Hsp70 proteins (reviewed in Refs. 9 and 10). Our previous studies revealed that mutation of this HPD sequence in ERdj3 to QPD reduced its ability to bind BiP in vivo and at the same time increased its binding to several substrates (30). We extended these experiments using two secretory pathway proteins that are unable to fold or be secreted when expressed alone: the immunoglobulin γ HC and the NS-1 κ LC. To discriminate the wild-type and mutant ERdj3 proteins, we produced untagged and HA-tagged versions of each, which were readily distinguished from each other by their mobility on SDS gels, and as expected, the untagged version of the proteins co-migrated with endogenous ERdj3 (Fig. 1). When each substrate was transiently expressed in COS-1 cells along with either wild-type or mutant ERdj3, we found that significantly more of the QPD mutant bound NS-1 κ LC (Fig. 1A) and the γ HC (Fig. 1B, lanes 2 and 4 in both immunoprecipitation panels) than the wild-type ERdj3 (lanes 1 and 5), although both ERdj3 proteins were expressed at similar levels, which was independent of the presence of the HA tag. When signals from multiple experiments were quantified, we found that under steady state conditions, the QPD mutant bound ~4 times more than the wild-type ERdj3 protein to these substrates when each protein was expressed alone. However, when wild-type and mutant ERdj3 were co-expressed with these substrates, the binding of mutant ERdj3 was similar (~1.2:1) to that of the wild-type protein (lanes 3 and 6). It is important to point out that these values were obtained from scanning multiple film images, which provides only limited quantitative information. Although endogenous ERdj3 is detected in the COS-1 monkey fibroblast cell line, we did not readily observe its binding to these two substrates in overexpression experiments (lane 7). It is possible that this represents differences in monkey ERdj3 substrate specificity or false impressions of the levels of endogenous ERdj3 expression when compared with that of transfected, human ERdj3 based on antibody reactivity.

Wild-type ERdj3 and the QPD Mutant Associate with Each Other to Form Heterodimers—The observation that ERdj3 and QPD bind similarly to substrates when both are co-expressed together led us to ask whether under these conditions the wild-type and mutant ERdj3 proteins might form mixed dimers. To determine whether this was occurring, we co-expressed an empty vector (Fig. 2A, lanes 1 and 2) or an HA-tagged version of ERdj3 (lanes 3 and 4) with untagged versions of either wild-type or QPD mutant ERdj3 and performed both an immunoprecipitation-coupled Western blotting assay (Fig. 2A) and a direct immunoprecipitation of metabolically labeled proteins (Fig. 2B). We found that HA-tagged ERdj3 readily co-precipitated the untagged form of both versions of ERdj3, demonstrating that mutation of the J domain had no effect on dimerization and that mixed dimers were readily formed. These associations were specific as the anti-HA antibody did not immunoprecipitate the untagged forms of these proteins (Fig. 2A, lanes 1 and 2 in the immunoprecipitation panel). Together, our findings raised the possibility that inclusion of wild-type ERdj3 as one subunit in heterodimers with mutant QPD ERdj3 could be inhibiting the increased steady state binding of the QPD mutant with these substrates.

Enhanced Substrate Binding of the ERdj3 QPD Mutant Is Dependent on Its Ability to Form Dimers—We next determined whether QPD mutants that were unable to form stable dimers...
retained their ability to bind substrates better than monomers of the wild-type ERdj3 protein. To render ERdj3 monomeric, we mutated Phe-326 to an aspartic acid (F326D) in both wild-type ERdj3 and the QPD mutant, which was previously shown to inhibit dimerization on the background of a wild-type J domain (16). As expected, neither protein could be co-immunoprecipitated with an HA-tagged form of ERdj3 in the presence and absence of the C-terminal 29 amino acids (Fig. 3A), nor were they directly isolated with the anti-HA antibody when expressed by themselves (Fig. 3B), demonstrating that indeed the F326D versions of both proteins no longer formed dimers. These mutants were compared with each other and with their dimeric forms for their ability to bind, γHC. Once again the largely homodimeric form of the QPD mutant showed much greater binding to the γHC than the homodimeric wild-type form under steady state conditions (Fig. 3C). However, when Phe-326 was mutated, the enhanced binding of the QPD mutant was diminished below that of wild-type ERdj3 dimers and to a level that was similar to a Phe-326 mutant with a wild-type J domain (Fig. 3C, compare lanes 3 and 5). Thus, the strong binding of the QPD mutant appears to be dependent on its ability to form dimers; in the absence of dimer formation, it no longer had a binding advantage. These results show that ERdj3 dimerization is essential for substrate binding, which occurs independent of ERdj3 interaction with BiP.

Construction of ERdj3 Mutants That Exclusively Form Homodimers—To examine the enhanced substrate binding of the QPD mutant more directly, we wished to produce wild-type and mutant ERdj3 proteins that would not form mixed dimers. Because Phe-326 in domain III is essential for dimer formation, we reasoned that replacing this residue with a cysteine might promote the formation of covalent homodimers, while at the same time, it would prevent the formation of heterodimers between a wild-type and mutant protein. Structural data on the closely related type I DnaJ-like protein Ydj1 revealed that the residue corresponding to Phe-326 is part of a hydrophobic cluster that mediates dimerization of the two Ydj1 protomers, which is further strengthened by interactions between the C-terminal α helix of one subunit with domain III of the other subunit (27). When we performed structural modeling of human ERdj3 based on the Type I DnaJ protein from T. thermophila (Fig. 4A) (32), we noticed that the 29 amino acids C-terminal of Phe-326 in ERdj3 could interfere with covalent dimerization and that deleting these residues might allow for better contact between the two Cys residues and thus improve disulfide bond formation between the two protomers. Thus, we mutated Phe-326 to Cys in wild-type ERdj3 and the QPD mutant, in the presence and absence of the C-terminal 29 amino acids of ERdj3 (F326C and F326CΔC, respectively).

We first examined the ability of the Cys-326 forms of these proteins to form covalent dimers using nonreducing SDS-PAGE (Fig. 4B). As our structural modeling indicated, the F326C mutants of these two proteins did not form detectable amounts of covalent dimers (Fig. 4B, lanes 4 and 5), but instead migrated as monomers just like the Phe-326 versions of these proteins that form noncovalent dimers (Fig. 4B, lanes 2 and 3). However, deletion of the 29-amino acid extension beyond domain III (ΔC) allowed the formation of readily detectable covalent homodimers for both the wild-type and the QPD J domain-containing proteins (Fig. 4B, lanes 6 and 7). The functionality of the various ERdj3 F326C mutants was examined next. Cells were co-transfected with NS-1 x kLC along with the various ERdj3 constructs, and their relative abilities to bind this
substrate were compared. The full-length ERdj3 F326C and QPD F326C mutants, which were unable to form detectable amounts of covalent dimers, were strongly impaired in substrate binding (data not shown), which is similar to that observed for the F326D mutant, suggesting that they were incapable of even forming noncovalent homodimers. Conversely, the two F326C ΔC proteins bound NS-1 κ LC similarly to their counterparts containing the wild-type Phe-326 (Fig. 4C, compare lanes 2 and 4 and lanes 3 and 5), demonstrating that the formation of covalent dimers did not significantly alter the substrate binding properties of either wild-type ERdj3 or the QPD mutant. Importantly, the F326C ΔC mutant did not form heterodimers with constructs possessing a phenylalanine at this position, nor was it immunoprecipitated directly with the monoclonal anti-HA antibody (Fig. 4D).

The Ability of QPD ERdj3 to Heterodimerize with Wild-type ERdj3 Is Essential for Reducing the Steady State Binding of the QPD Mutant with Substrates—Because the QPD F326C ΔC mutant formed dimers that bound to substrate similarly to the ERdj3 QPD mutant, and at the same time to circumvent the formation of any heterodimers with the endogenous ERdj3 protein, we used this mutant to revisit the experiment in which wild-type ERdj3 and the QPD mutant were co-expressed. When the wild-type and mutant proteins were unable to form heterodimers, co-expression of the wild-type protein no longer dramatically reduced binding of the QPD mutant to either the NS-1 κ LC or the γ HC (Fig. 5, A and B, compare lanes 5 and 6). This argues that the effects of wild-type ERdj3 on the QPD mutant must be primarily exerted via its ability to form heterodimers, which results in the QPD mutant appearing more like the wild-type protein in terms of substrate interaction.

Substrates Influence the Kinetics of Wild-type ERdj3 Release, whereas ERdj3 QPD Homodimers Are Uniformly Defective in Substrate Release—It was unclear whether the enhanced steady state binding of QPD mutant homodimers to substrates was due to a higher affinity for these substrate or whether this was a result of impaired release, the latter of which is more in keeping with previous in vitro experiments performed with ERdj3, suggesting that in vitro substrate affinities were not significantly different for these two proteins (21). To address this, we conducted a pulse-labeling experiment in which NS-1 κ LC was co-expressed with wild-type ERdj3, the QPD F326C ΔC mutant, or a combination of the two. When the ERdj3 constructs were co-expressed with NS-1 κ LC alone, even after a relatively short labeling time, the QPD mutant bound slightly better to the NS-1 κ LC (Fig. 6A, compare lanes 2 and 4 in the anti-κ LC immunoprecipitation panel), although the difference was much less dramatic than the steady state binding observed in the Western

FIGURE 4. Development of ERdj3 mutants that form restricted homodimers. A, model of ERdj3 was generated based on the structure of DnaJ from T. thermophilus (Protein Data Bank code 4J80). One protomer of the dimer is shown in green, and the other one is shown in blue. The Phe-326 residues that are required for dimer formation are illustrated in red, and the helical C-terminal helical extension, which also contributes to dimer stability, is shown in gray. The black square represents a zoom of the area surrounding Phe-326 with a substitution of cysteine for this residue (yellow) and in which the C-terminal helical extension has been removed. This illustrates how such a deletion allows for increased flexibility of the Cys side chains, increasing their ability to interact and form a disulfide bond. B, COS-1 cells were transfected with wild-type or QPD mutant ERdj3 (U3), versions of these proteins in which Phe-326 is mutated to Cys (F326C), or versions of the Phe-326 mutants in which the C-terminal helical extension was removed (F326C ΔC). Twenty-four hours later, cells were lysed, and whole cell lysates were analyzed by nonreducing SDS-PAGE followed by immunoblotting with anti-ERdj3 antiseraum. Bands representing ERdj3 monomers and dimers are indicated. Hsc70 was used as loading control. C, COS-1 cells were co-transfected with the indicated ERdj3 constructs and NS-1 κ LC. Twenty-four hours later, cells were cross-linked with DSP followed by lysis with Nonidet P-40 buffer and immunoprecipitated with anti-κ LC (α κ LC IP). A fraction of the whole cell lysates (input) and immunoprecipitated material was analyzed by reducing SDS-PAGE followed by immunoblotting with the indicated antibodies. Hsc70 was used as a loading control for the input. D, top, COS-1 cells were co-transfected with HA-tagged versions of wild-type or QPD mutant ERdj3 along with untagged wild-type ERdj3 or the QPD or QPD F326C ΔC mutants. Twenty-four hours later, cells were labeled with [35S]methionine/cysteine for 3 h. Cell lysates were prepared and immunoprecipitated (IP) with the indicated antibodies. Samples were analyzed by SDS-PAGE followed by autoradiography. Bottom, COS-1 cells were transfected with the untagged QPD F326C ΔC mutant, immunoprecipitated with the indicated antibodies after labeling and lysis, and analyzed as in the top panel.
Ex Vivo Regulation of ERdj3-Substrate Binding and Release

FIGURE 5. Substrate binding by the QPD ERdj3 mutant that forms restrictive homodimers is not reduced by wild-type ERdj3 co-expression. A and B, COS-1 cells were transfected with the indicated HA-tagged and untagged versions of ERdj3 (JS) along with BIP and either the γ HC (A) or the NS-1 κ LC (B). Twenty-four hours later, cells were cross-linked with DSP followed by lysis with Nonidet P-40 buffer and immunoprecipitated with protein A (Pro-A IP) (A) or an anti-κ LC antiserum (α κ LC IP) (B). A portion of the whole cell lysates (Input) and immunoprecipitated proteins was then subjected to reducing SDS-PAGE analysis followed by Western blotting with the indicated antibodies. Hsc70 was used as loading control.

no indication of increasing. These results demonstrate two things. First, the fact that the signal for wild-type ERdj3 decreased, whereas that of the QPD F326C ΔC mutant remained stable, suggests that ERdj3 does not undergo reiterated cycles of binding to these substrates. Second, the rate of release of wild-type ERdj3 was not the same for both of these substrates. Instead we found that in the case of the short-lived NS-1 κ LC, the release of wild-type ERdj3 was fairly quick, whereas for the longer-lived γ HC, decreased binding of wild-type ERdj3 was much slower, suggesting that the affinity of ERdj3 for a substrate may affect release.

DISCUSSION

Models for Hsp70/DnaJ interactions with substrates based on genetic and biochemical studies with bacterial and eukaryotic orthologues posit that substrates are captured first by DnaJ proteins and then transferred to the ATP-bound form of the Hsp70 protein. In vitro studies demonstrate that the concomitant stimulation of the ATPase activity of the Hsp70 enhances its affinity for the substrate and signals the release of the DnaJ protein (33–35). However, the details of the DnaJ binding cycle occurring in cells are less well delineated. Studies described here address the in vivo requirements for both substrate binding and release for an ER-localized type I DnaJ protein, ERdj3. We previously reported that mutation of Phe-326 to either Asp or Ala inhibited ERdj3 dimer formation in vitro and disrupted its ability to bind substrates in vivo (16). In this study, we extended these observations to show that ERdj3 F326D, which remains monomeric, is unable to bind substrates in mammalian cells. A J domain mutant of ERdj3 (QPD) that interferes with the ability of ERdj3 to stimulate the ATPase of BiP (30) was found to be deficient in transferring a substrate from ERdj3 to BiP in vitro (21). However, when this was examined in cells, we found that although the QPD mutant bound to the two substrates examined in this study at considerably higher levels than wild-type ERdj3, when each was expressed alone, there was no compensatory decrease in BiP binding (Figs. 1 and 5). It is possible that the presence of other ER-localized DnaJ family members at the translocon are sufficient to target BiP to these substrates in vivo.

The enhanced steady state binding of the QPD mutant was not due to an increased affinity for substrates, but instead we found that this mutation impaired the release of ERdj3 from substrates in cells. Importantly, we found that mutation of F326D on the QPD background decreased substrate binding to about the same level as that seen for a monomeric ERdj3 mutant (F326D) that possessed a wild-type J domain. This strongly suggests that dimerization of ERdj3 is required for initial substrate binding in vivo, which is independent of the ability of ERdj3 to interact productively with BiP via a functional J domain. In a previous study, both substrate-binding and dimerization mutants of ERdj3 were equally impaired in rescuing the growth defect in yeast cells lacking both Ydj1 and Hlj1 protein functions, whereas expression of both wild-type ERdj3 and the QPD mutant of ERdj3 restored normal growth (36). As data presented here demonstrate that the ERdj3 dimerization mutant is unable to bind substrates in vivo, the yeast data suggest that direct substrate binding can be more critical to some DnaJ family functions than serving as a co-factor for BiP or...
Hsp70-regulated folding. It is noteworthy that for other DnaJ family members, the converse is true; stimulating the ATPase of an Hsp70 is critical, but substrate binding is not (37). More recently, a group of type IV family members have been identified that possess a J domain without the highly conserved HPD motif and whether or not they can interact with BiP to stimulate its ATPase activity is a de facto monomer in its association with the substrate, for which our data demonstrate dramatically reduced substrate association, which could lead to release of ERdj3. It will be interesting to determine whether indeed a second ERdj3-BiP-binding site can be localized to the charged amino acids at the C terminus of BiP and whether or not they are crucial for releasing ERdj3 from substrates in vivo.

The QPD F326C ^SC mutant that forms exclusive homodimers provided us with a tool to determine whether ERdj3 undergoes reiterative cycles of binding to substrates. When this mutant was co-expressed with wild-type ERdj3 and either the short-lived NS-1 κ LC or the much longer-lived γ HC, we found that although both forms of ERdj3 initially bind these substrates at similar levels, wild-type ERdj3 binding decreased over time, whereas that of QPD F326C ^SC remained constant. This similar function in terms of J protein interaction with BiP. In agreement with this idea, it has been shown that ERdj3 can induce an open lid conformation in BiP, even if the ERdj3 protein comprises the QPD mutation, arguing for a second binding site between BiP and ERdj3 (47). However, the issue remains as to how an Hsp70 monomer can release a DnaJ dimer from substrates. The data presented here allow us to propose a possible mechanism. We found that only a single functional J domain that can interact with BiP to stimulate its ATPase activity is required to induce release of the ERdj3 dimer from substrates as the QPD mutant bound to substrates at a level similar to that of wild-type ERdj3 when it was part of a WT/QPD heterodimer, whereas QPD homodimers bound substrates much more stably. This suggests that the interaction between the nucleotide-binding domain of BiP and the J domain of ERdj3 either is most critical to the process or happens first. This interaction would be followed or complemented by the acidic C-terminal region of the Hsp70 protein inserting into the peptide-binding pocket of one of the DnaJ monomers, helping to displace it from the substrate. As a result, the second protomer of the DnaJ protein would be rendered a de facto monomer in its association with the substrate, for which our data demonstrate dramatically reduced substrate association, which could lead to release of ERdj3. It will be interesting to determine whether indeed a second ERdj3-BiP-binding site can be localized to the charged amino acids at the C terminus of BiP and whether or not they are crucial for releasing ERdj3 from substrates in vivo.

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would argue that when QPD ERdj3 is expressed by itself, the half-life of substrate should increase, which we have observed.\textsuperscript{6} There was no evidence of an increase in the pool of substrate bound to the QPD mutant, arguing against reiterative cycles of binding. These data, as well as our previous published results, suggest that QPD may be acting as a holdase to prevent substrate aggregation although it cannot promote substrate unfolding (21, 36).

It is intriguing that the kinetics of ERdj3 release was slower for the long-lived substrate than for the more rapidly degraded substrate. It is possible that the degradation of substrates may be dependent on their transfer to other ERdj proteins. Both ERdj4 and ERdj5 (48, 49) are involved in the degradation of unfolded ER proteins, and we have found that both ERdj4 (50) and ERdj5\textsuperscript{6} bind to NS-1 and LC and γ HC. A previous study by our laboratory reported that BiP did not appear to undergo repetitive binding to the γ HC in cells (50). It is tempting to speculate that the relatively slow release of ERdj3 we observed from this substrate in vivo is responsible for stabilizing the binding of BiP. In keeping with this possibility, \textit{in vitro} experiments revealed that the inclusion of ERdj3 in reactions containing BiP and the purified C\textsubscript{\gamma1} domain of HC dramatically reduced the ATP-mediated release of BiP from the C\textsubscript{\gamma1} domain (47).

In summary, the data reported here demonstrate that ERdj3 binds unfolded substrates \textit{in vivo} only as a dimer regardless of whether it is able to functionally interact with BiP. The release of ERdj3 from substrates requires that the J domain of only one protomer of the dimer interacts productively with BiP to stimulate its ATPase activity. We find that ERdj3 does not undergo reiterative cycles of binding to even a long-lived ER-associated protein degradation substrate, which may allow transfer to other BiP co-factors to aid in the degradation of such substrates.

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