Inhibition of immunoglobulin folding and secretion by dominant negative BiP ATPase mutants

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ABSTRACT A group of resident ER proteins have been identified that are proposed to function as molecular chaperones. The best characterized of these is BiP/GRP78, an hsp70 homologue that binds peptides containing hydrophobic residues in vitro and unfolded or unassembled proteins in vivo. However, evidence that mammalian BiP plays a direct role in protein folding remains circumstantial. In this study, we examine how BiP interacts with a particular substrate, immunoglobulin light chain (λ LC), during its folding. Wild-type hamster BiP and several well-characterized BiP ATPase mutants were used in transient expression experiments. We demonstrate that wild-type λ LCs showed prolonged association with mutant BiP which inhibited their secretion. Both wild-type and mutant BiP bound only to unassembled and partially folded LCs. The wild-type BiP was released from the incompletely folded LCs, allowing them to fold and be secreted, whereas the mutant BiP was not released. As a result, the LCs that were bound to mutant BiP were unable to undergo complete disulfide bond formation and were retained in the ER. Our experiments suggest that LCs undergo both BiP-dependent and BiP-independent folding steps, demonstrating that both ATP binding and hydrolysis activities of BiP are essential for the completion of LC folding in vivo and reveal that BiP must release before disulfide bond formation can occur in that domain.

Nascent proteins that enter the endoplasmic reticulum (ER) must fold and assemble into multimeric complexes in an oxidizing milieu that contains millimolar concentrations of protein and calcium (1). In the past decade, proteins that participate transiently with incompletely folded and assembled proteins have been identified and characterized. Some of these associated proteins are enzymes responsible for glycosylation, disulfide bond formation, and isomerization of peptide bonds (1). Others, termed “molecular chaperones,” appear to stabilize protein folding intermediates and are thought to prevent incorrect interactions between protein domains or promote correct ones (1, 2). The hsp70 family of proteins are the most abundant and best characterized of the molecular chaperones. They are present in all cellular organelles and in all organisms. BiP is identical to a 78-kDa glucose-regulated protein, grp78 (3–5) and is the ER member of the hsp70 family. The evidence for the role of BiP in protein folding is largely circumstantial. It was first identified stably and noncovalently associated with the unassembled, nontransported Ig heavy chains in Abelson virus transformed pre-B cell lines (6). BiP also associates transiently with nascent proteins that are often unassembled and may be incompletely folded (7–10). BiP also binds more stably to mutants proteins that are in many cases thought to be incorrectly folded (11, 12). Peptides containing hydrophobic amino acids are the most effective stimulators of BiP’s ATPase activity (13). Affinity screening of two peptide display libraries revealed that BiP preferentially binds peptides containing hydrophobic amino acids in every other position, which would place them on one side of an extended polypeptide chain (14). Together, these studies are consistent with the possibility that BiP binds to hydrophobic stretches on nascent or unassembled polypeptides that would become inaccessible after folding and assembly are complete. In vitro folding studies with dnaK (15), the bacterial hsp70 homologue, and human hsp70 (16), a cytosolic family member, demonstrate that these chaperones bind to unfolded or denatured proteins and, in concert with other chaperones, promote their correct folding. Most recently, the folding and oxidation of the yeast vacuolar protein p1CPY was inhibited when dominant negative Kar2 (BiP) mutants were expressed (17). Although the Kar2 mutants have not been characterized biochemically, the mutations map to the ATP binding domain and are assumed to inhibit nucleotide binding or hydrolysis. It remains to be shown that the in vivo binding of mammalian BiP to unfolded proteins in any way affects their folding. Although yeast and mammalian BiP share a high degree of sequence homology and are thought to perform some of the same functions, it is increasingly clear that mammalian BiP does not do everything that yeast BiP does. Therefore, determining which functions they share is important to understanding which of the functions attributed to yeast are actually general characteristics of hsp70 family members.

All hsp70 proteins bind and hydrolyze ATP, and ATP is required for the in vitro folding of proteins by dnaK (15) and hsp70 (16). This folding is thought to occur through ATP-driven cycles of binding and release from substrate proteins. BiP, like other family members, can be released from target proteins in vitro with ATP (3, 18). Recent experiments have established that ATP binding, but not hydrolysis, is required for the in vivo release of dnaK and hsp70 from proteins (19, 20). It has been hypothesized that protein folding occurs during the release phase of hsp70 binding. If folding does not occur, hsp70 would rebind and prevent aggregation of the protein. Another cycle of ATP-mediated release would provide another opportunity for folding. However, direct data for cycles of BiP binding and release or for BiP release to occur before protein can fold have not been obtained.

To investigate the role of BiP in the in vivo folding of proteins, we have examined its interactions with murine immunoglobulin lambda light chains (λ LCs). In vivo, newly synthesized LCs interact transiently with several molecular chaperones, including BiP and GRP94 (6, 21, 22). λ LCs were transiently coexpressed with a series of hamster BiP mutants with well-characterized point mutations in the ATP binding domain. These mutations affect the ability of the BiP mutants

Abbreviations: ER, endoplasmic reticulum; LC, light chain; NEM, N-ethylmaleimide; DTt, dithiothreitol; hsp70, 70-kDa heat shock protein.

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to bind ATP, to undergo an ATP-mediated conformational change, or to hydrolyze ATP (20). All of these mutants are able to bind substrate proteins in vivo (23, 24) and in vitro (20, 23), but the ATP binding and conformational change mutants are unable to release bound proteins in vitro when ATP is added to isolated BiP–protein complexes (20, 23). The folding status of LCs bound to both wild-type and mutant BiP was determined, and the effects of the BiP mutants on LC release from BiP in vivo, disulfide bond formation of the individual LC domains, and secretion of the LCs were assessed.

**MATERIALS AND METHODS**

**LC and BiP DNA Clones.** DNAs encoding either wild-type ΔI LC (25) or a nonsecreted ΔLC point mutant, DK82, in which aspartic acid at position 82 in the Vᵡ domain is mutated to lysine (S.A., J.M., J.D., and Y.A., unpublished data) were used. A hamster BiP cDNA clone was obtained from Amy S. Lee (University of Southern California, Los Angeles) and was placed under the control of an adenovirus promoter in the pMT vector which contains an simian virus 40 origin of replication. Single point mutations in the BiP cDNA clone have been made and characterized previously (20, 23). The T229G mutant is defective in ATP hydrolysis, the T37G mutant is unable to undergo an ATP-induced conformational change, and the G227D mutant does not bind ATP (20). All BiP mutants are in the pMT vector.

**Transient Expression of ΔLCs with Hamster BiP.** COS monkey fibroblasts were transfected with DNAs for either wild-type ΔLCs or DK82 mutant ΔLCs alone, or together with either wild-type or mutant BiP using the DEAE-dextran procedure as described (23). Cells were analyzed 40 h post-transfection.

**Dithiotreitol (DTT) Washout Experiments.** The treatment of cells with DTT is an adaptation of the protocol described by Braakman et al. (26). Transfected cells were incubated with 10 mM DTT for 45 min to prevent disulfide bond formation in newly synthesized proteins and either analyzed immediately or washed twice with phosphate-buffered saline (PBS) and reincubated in complete medium for various times before analysis. At the indicated time, the cells were washed once with PBS containing 20 mM N-ethylmaleimide (NEM) and then lysed in a lysis buffer consisting of 50 mM Tris (pH 7.5), 150 mM NaCl, 0.5% Nonidet P-40, and 0.5% deoxycholate to which 20 mM NEM and 10 units of apparse (Sigma) were added.

**Metabolic Labeling and Immunoprecipitation.** For LC secretion experiments, 100-mm dishes of cells were metabolically labeled with 100 μCi (1 Ci = 37 GBq) of [35S]Translabel (ICN) for 3 h. Labeled cells were lysed in the presence of apparse, cleared by centrifugation, and then immunoprecipitated with rodent-specific anti-BiP antiserum (24), goat anti-mouse ΔLC antiserum (Southern Biotechnology Associates), or protein A Sepharose (Pharmacia) as described (24). Samples of culture media were immunoprecipitated with anti-Δ. For folding studies, cells were incubated in methionine- and cysteine-free media for 30 min and then pulse-labeled with 100 μCi [35S]Translabel for 45 min in the presence of 10 mM DTT. Cells were lysed immediately as described above or washed and reincubated in complete media for the indicated times. In both cases NEM and apparse were added to the lysis buffer. Cell lysates were immunoprecipitated as described above. Proteins were electrophoresed on SDS/polyacrylamide gels and visualized by autoradiography.

**Western Blot Analysis.** Unlabeled COS cell transfectants were treated with DTT as described. Proteins were immunoprecipitated with either anti-Δ or the rodent-specific anti-BiP antiserum and then separated on 10% SDS/polyacrylamide gels under nonreducing conditions. Proteins were transferred to nitrocellulose and probed with the anti-Δ antiserum, followed by incubation with HRP-protein A and visualized by ECL (Amersham).

**RESULTS**

**Effects of BiP Mutant Expression on the Secretion of ΔLCs.** To assess the role of BiP in protein folding in vivo, we characterized its interactions with murine ΔLCs. Wild-type or mutant BiP was expressed in COS monkey fibroblasts along with either wild-type ΔLCs or a nonsecreted ΔLC point mutant, DK82. The wild-type ΔLCs were not readily detected in association with wild-type BiP in coimmunoprecipitation experiments and were secreted into the culture medium (Fig. 1A). However, when we coexpressed wild-type ΔLCs with two different BiP ATPase mutants (T229G or T37G), a significant proportion of the ΔLCs were coprecipitated with the BiP mutants and fewer ΔLCs were secreted relative to the control cultures (Fig. 1A). Because endogenous wild-type BiP is also present in the cells transfected with BiP mutants, we propose that the inhibition of LC secretion must be due to BiP mutants competing with endogenous BiP for the LC and acting as "chaperone traps" that capture and retain their targets.

When the LC mutant DK82 was similarly analyzed, readily detectable amounts of wild-type BiP coprecipitated with the ΔLCs (Fig. 1B) due to the mutation in the Vᵡ domain. Expression of DK82 with mutant BiP resulted in a further increase in the amount of BiP coprecipitating with the ΔLCs (Fig. 1B), showing that wild-type BiP binding to these mutant ΔLCs is not saturating. The increased association of mutant BiP with these ΔLCs could either represent stabilized binding to the same site that wild-type BiP normally binds to or it could be a result of more mutant BiP molecules binding per LC. BiP was previously shown to be released from other LCs prior to their degradation and to rebinding to nascent ΔLCs (8, 27). If wild-type BiP normally undergoes cycles of binding and release to
substrates and the BiP mutants bind but cannot release (20, 23), a net increase in mutant BiP binding, such as the one seen here, would be observed.

**Effects of Prolonged Association with BiP on the Folding of λ LCs.** As a first step toward distinguishing between these possibilities, we examined the folding status of the LCs coexpressed with either wild-type or mutant BiP using the oxidation state of the LCs to monitor their folding. λ LCs are composed of a variable antigen-binding domain (Vλ) and a constant domain (Cλ), each of which folds into a compact structure consisting of two twisted β sheets stabilized by a single disulfide bond (28). In vitro, each domain is able to fold autonomously (29, 30). COS cell transfectants were metabolically labeled in the presence of DTT to prevent disulfide bond formation in newly synthesized LCs, and then chased in the absence of DTT. In vivo DTT treatment is fully reversible and has been used to investigate folding intermediates of several proteins (26, 31) including LCs (32). Importantly, DTT should have no effect on BiP, because it contains no disulfide bonds. Wild-type LCs were completely reduced upon DTT treatment (F0, Fig. 2A, time = 0), and when DTT was removed from cells coexpressing wild-type BiP, the LCs completely oxidized within 1 h (Fox, Fig. 2A). However, in cells coexpressing the BiP mutants (only T37G is shown), only half of the LCs became fully oxidized, while the other half folded to a form with an intermediate mobility (F1, Fig. 2A, time = 1). Interestingly, there was no evidence that mutant BiP expression inhibited the conversion of the fully reduced form of LC to the partially oxidized form, demonstrating that one domain could fold and undergo disulfide bond formation independent of BiP.

We similarly examined the DK82 mutant and found that even in the presence of wild-type BiP only a minor fraction of these LCs reached the Fox form after 1 h (Fig. 2A). Coexpression of DK82 with mutant BiP practically eliminated the formation of Fox but again had no discernable effect on the conversion of F0 to F1 (Fig. 2A). These data demonstrate three things. (i) Some folding and disulfide bond formation occur while the LC is bound to BiP generating the F1 form. Presumably this represents autonomous folding of a domain not bound to BiP. (ii) Both mutant BiP and a point mutation in Vλ affect the same LC folding step. This suggests that BiP primarily mediates the folding of Vλ and not Cλ and supports earlier observations that mutations in Vλ affect BiP binding (33, 34). (iii) These data are more consistent with the hypothesis that the increased association of DK82 with mutant BiP (Fig. 1B) represents the stable binding of mutant BiP to a site that wild type normally cycles on and off of and not due to additional mutant BiP molecules interacting with multiple sites on the same LC (e.g., Cλ domain).

To determine whether F1 represents a normal LC folding intermediate, we performed DTT washout experiments on unlabeled COS transfectants and followed the folding of wild-type LC expressed with either wild-type or mutant BiP by Western blot analyses. The majority of the LCs were fully reduced at t = 0 (Fig. 2B), although some preexisting LCs were apparently not susceptible to DTT reduction. In the presence of wild-type BiP, ~80% of the reduced LCs became completely oxidized over the course of the 1-h chase (Fig. 2B and C). An intermediate form of LC that chased to Fox was clearly evident. When the LCs were coexpressed with mutant BiP, the F1 form disappeared with the same kinetics as observed with wild-type BiP (Fig. 2C), but the F1 form remained stable throughout the 1-h chase (Fig. 2B and C). In short, at least three folding forms of LC are detectable: LC containing no (F0), one (F1), and two (Fox) disulfide bonds that probably represent LC with neither, one, and both Ig domains folded, respectively. Mutant BiP expression does not affect the folding
of the completely reduced LC to the F₁ form, but does inhibit the folding of F₁ to F₀x.

**Examination of the Folding Status of BiP-Bound LCs.** To determine which LC folding intermediates bind BiP, we used the DTT washout protocol to examine the oxidation state of those LCs that coprecipitated with wild-type or mutant BiP. In biosynthetic labeling experiments, we found that wild-type BiP bound to the F₀ form of LC in a nearly stoichiometric manner, but failed to bind the F₀x form that is present after a 1-h chase (Fig. 3A). It was difficult to assess binding of wild-type BiP to the F₁ form since there was so little of it present at the 1-h chase. When the same experiments were done on the LCs expressed with mutant BiP, we found that mutant BiP bound to the F₀ form of LC at t = 0. After a 1-h chase about half of the LCs were partially oxidized and the other half were completely oxidized, which is consistent with the previous data. However, mutant BiP was only detected with the F₁ form of LC and not with the F₀x form (Fig. 3A).

In an attempt to detect more LCs in the F₁ form when they are expressed with wild-type BiP, we performed a similar experiment on unlabeled cell lysates and detected LC by Western blot analyses. COS cells expressing LCs alone, or LCs with either wild-type or mutant BiP, were examined. The LC expressed alone folded completely during the 1-h chase and could not be precipitated with antibodies to BiP (Fig. 3B). The majority of the LCs expressed with wild-type hamster BiP folded completely, but a small amount of LC are still present in the F₁ form which can be precipitated with antibodies to BiP (Fig. 3B). Readily detectable amounts of both the F₁ and F₀x forms of LC are observed with mutant BiP expression, and again, only the F₁ form is complexed with BiP. Similar experiments were performed on the DK82 LCs, and we found that both wild-type and mutant BiP bound to the F₀ and F₁ forms. However, the small amount of F₀x that forms in the presence of wild-type BiP was not associated with BiP (data not shown). Together, these experiments demonstrate that both wild-type and mutant BiP bind to unfolded and folding intermediates of LCs. This binding does not inhibit the folding and oxidation of either of the domains when wild-type BiP is expressed. Interestingly, even though a large portion of the LC are initially bound to the BiP mutant (Fig. 3A), there is no evidence that this binding has any effect on the oxidation of one of the LC domains. It appears that the wild-type λ LC turn over faster in the presence of mutant BiP (Figs. 2 and 3). The DK82 LC have a shorter half-life than their wild-type counterparts, but it is not further shortened in the presence of mutant BiP. Together, these data suggest that the unfolded LC are better substrates for degradation.

**Requirements for ATP Hydrolysis in the in Vitro Folding and Secretion of LCs.** The in vitro release of dnak and hsc70 from substrate proteins requires ATP binding but not ATP hydrolysis (19). Recently, using our BiP ATPase mutants, we confirmed this finding and extended it by demonstrating that ATP binding followed by an ATP-induced conformational change in BiP is sufficient for protein release in vitro, and that ATP hydrolysis is not necessary (20). We next examined the effect of the three classes of BiP mutants on LC folding and secretion. COS cells were transfected with wild-type λ LC alone or together with wild-type BiP, G227D (ATP binding mutant), T37G (ATP-induced conformational change mutant), or T229G (ATP hydrolysis mutant). Forty hours post-transfection, DTT washout experiments were performed, and LC folding status was determined by western blotting. All three classes of mutants retarded the complete folding of LCs equally, and only fully oxidized LC were secreted (Fig. 4). Evidently, the stable binding of BiP mutants to the F₁ form of LC prevented its secretion (Fig. 4), which explains the secretion block data presented in Fig. 1A.

**DISCUSSION**

LCs can usually be secreted without Ig heavy chains. Thus, unlike its effect on free heavy chains (7, 35), BiP does not block the secretion of free LCs. However, the ectopic expression of BiP ATPase mutants resulted in prolonged binding of LCs to the mutant BiP and an inhibition of LC secretion. By investigating the status of proteins bound to wild-type and mutant BiP, it was possible to better understand the role of BiP in protein folding. First, we observed that both wild-type and mutant BiP bound only to the unoxidized or partially oxidized forms of LCs, but that even with the BiP mutants, there was...
no evidence of BiP binding to the completely oxidized LC. This is in keeping with studies from other laboratories that suggest that BiP preferentially binds to unfolded or oxidized proteins (8-10, 36). In a recent study, Knittler et al. (10) found that LCs bound to BiP were incompletely oxidized, whereas lysis of the cell in the presence of ATP resulted in BiP release and the isolated LCs were more fully oxidized. However, none of these studies could distinguish between protein folding causing the release of BiP or whether folding occurred only after release from BiP. Our data provide evidence that proteins must be released from BiP for complete folding to occur. This is most compatible with BiP binding to unfolded regions to prevent their aggregation, and then releasing the proteins to allow them to fold. Our data with mammalian BiP mutants are compatible with that obtained recently with yeast BiP mutants (17), suggesting that this represents a general mechanism for hsp70-assisted protein folding in the ER.

Interestingly, the LCs bound to either wild-type or mutant BiP underwent some folding as measured by the formation of one disulfide bond. Because our in vitro data show that at least two of these BiP mutants are unable to be released from bound proteins (20), we theorize that the folding of one of the LC domains occurs while the protein is bound to BiP. The most logical explanation is that this domain does not associate with BiP and folds independently. This is further supported by the studies on the DK82 LC mutant. These LCs have a mutation in the variable domain that caused them to exhibit increased binding to wild-type BiP and not to be secreted. Examination of the folding status of these LC mutants revealed that only one of their domains folded efficiently. Binding of mutant BiP had no effect on the conversion of the completely reduced form of DK82 to the partially oxidized form, suggesting that both the DK82 mutation and BiP affect the folding of the same domain. These data are consistent with the hypothesis that BiP normally binds to the variable domain of the LC while it is unfolded, and only after BiP is released will this domain fold. Mutational analyses of the cysteine residues in both domains need to be performed to determine which LC domain BiP binds.

Although we have concluded that mutant BiP binds to the same domain of the DK82 LCs that wild-type BiP binds to, it was clear from our experiments that more mutant BiP was coprecipitated with these LCs than wild-type BiP. If wild-type BiP binding to mutant proteins is unstable, this increased association could only occur if multiple mutant BiP molecules were binding to a single LC domain. Conversely, if “stable” BiP binding to mutant proteins actually occurs through continual cycles of release and rebinding, we would anticipate that the increased binding was due to the stabilization of a single site. Serial immunoprecipitation experiments revealed that most of the DK82 mutant LCs coexpressed with mutant BiP were precipitated by the anti-BiP antibody when this antibody was used in excess, whereas much less of the DK82 LCs expressed with wild-type BiP were coprecipitated with BiP (data not shown). This finding, together with the fact that mutant BiP does not further inhibit the folding of this LC, suggests that the increased association of mutant BiP with DK82 LCs occurs because the BiP mutants, unlike wild-type BiP, are unable to undergo cycles of ATP-mediated binding and release. The hypothesis that BiP and other hsp70 proteins work through cycles of binding and release has been proposed for some time. Furthermore, experimental data show that once BiP is released from a protein it can be reused to bind another protein (27). Thus, we feel it is very probable that BiP binds to nascent proteins, releases them, and if they fail to fold while released, BiP will rebind to them. Therefore, the term “continuous binding” is probably more accurate than “stable binding” when describing the association of mutant proteins with BiP.

Several ER resident proteins appear to act as molecular chaperones, and it has been unclear as to whether these proteins act together, sequentially, or independently to promote protein folding in the ER, and their relationship to the ER enzymes that catalyze and stabilize protein folding is not well understood. Recent studies have begun to address this question and the data suggest that there may be sequential interactions between BiP and either grp94 (8) or calnexin (9, 37). In this study, we examined the folding of LCs by assaying their oxidation status. Disulfide bond formation stabilizes folding and is catalyzed by the resident ER enzyme, protein disulfide isomerase (38). The finding that BiP must release from the LC before disulfide bond formation occurs in the second domain suggests sequential functions of a chaperone and a folding enzyme.

Perhaps the most surprising finding came when we examined the effect of the various classes of BiP ATPase mutants on protein folding and secretion. Our previous studies confirmed that ATP hydrolysis was not required for BiP release in vitro, but that an ATP-induced conformational change in BiP was sufficient (20). However, in vivo the ATP hydrolysis mutant was as unable to promote LC folding as the ATP binding or conformational change mutants. This suggests that either release is not sufficient to allow folding or that ATP hydrolysis is required for release in vivo. At this point, we cannot distinguish between these possibilities. The requirement for ATP hydrolysis in the in vivo function of HSP70 proteins is supported by the finding that a dnaK hydrolysis mutant acts as a dominant negative mutant in bacteria cells (39), even though it can be released from substrate proteins in vitro with ATP (19).

These experiments demonstrate that mammalian BiP is integrally involved in chaperoning protein folding in the ER and importantly, provide insights into the mechanism. By using ATPase BiP mutants that are incapable of dissociation, we have shown that fully reduced LCs can form one disulfide bond while bound to BiP. Presumably this represents autonomous folding of the Ig domain that is not associated with BiP. However, LCs are unable to complete their folding when bound to mutant BiP that lacks ATPase activity, demonstrating that complete folding cannot proceed without BiP’s ATPase activity and/or its release. The profound inhibition of folding and secretion, even in the presence of endogenous wild-type BiP, suggests that most, if not all, of the LCs associate with BiP during folding. Finally, our data strongly suggest that multiple cycles of BiP binding and release occur, providing an increased opportunity for the mutant BiP to bind and act as a “chaperone trap” to halt the folding process.

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5274  Cell Biology: Hendershot et al.