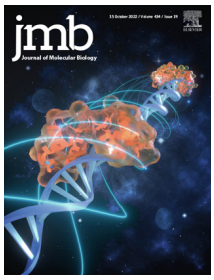
Research Article



Grp94 Works Upstream of BiP in Protein Remodeling Under Heat Stress

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Abstract

Hsp90 and Hsp70 are highly conserved molecular chaperones that promote the proper folding and activation of substrate proteins that are often referred to as clients. The two chaperones functionally collaborate to fold specific clients in an ATP-dependent manner. In eukaryotic cytosol, initial client folding is done by Hsp70 and its co-chaperones, followed by a direct transfer of client refolding intermediates to Hsp90 for final client processing. However, the mechanistic details of collaboration of organelle specific Hsp70 and Hsp90 are lacking. This work investigates the collaboration of the endoplasmic reticulum (ER) Hsp70 and Hsp90, BiP and Grp94 respectively, in protein remodeling using in vitro refolding assays. We show that under milder denaturation conditions, BiP collaborates with its co-chaperones to refold misfolded proteins in an ATP-dependent manner. Grp94 does not play a major role in this refolding reaction. However, under stronger denaturation conditions that favor aggregation, Grp94 works in an ATP-independent manner to bind and hold misfolded clients in a folding competent state for subsequent remodeling by the BiP system. We also show that the collaboration of Grp94 and BiP is not simply a reversal of the eukaryotic refolding mechanism since a direct interaction of Grp94 and BiP is not required for client transfer. Instead, ATP binding but not hydrolysis by Grp94 facilitates the release of the bound client, which is then picked up by the BiP system for subsequent refolding in a Grp94-independent manner.

Introduction

Grp94 and BiP are two of the most abundant molecular chaperones in the endoplasmic reticulum (ER). In the ER, Grp94 is essential for the proper assembly and maturation of secretory and membrane proteins such as Toll-like receptors (TLRs), integrins, and insulin-like growth factors (IGFs).[1] Grp94 plays an essential role in metazoans and the knockdown of Grp94 results in delayed development in multiple species.[2] Some clients (substrates) of Grp94 are involved in cancer development and progression, and Grp94 overexpression has been observed in multiple cancers.[3] Unlike Grp94, which has a relatively short clientele list, BiP interacts with and ensures the correct folding of newly synthesized proteins targeted to the ER.[4] Additionally, BiP targets misfolded proteins for degradation.[5] Similar to Grp94, protein remodeling by BiP has been linked to proliferation, invasion and metastasis of cancers, and was also shown to promote resistance against chemotherapeutics.[6] Together, this highlights the importance in understanding the fundamental mechanisms of

ER chaperones, which could aid in treating or preventing these conditions by specifically targeting chaperones or chaperone interactions.

Grp94 and BiP are ATP-dependent chaperones that belong to the highly conserved heat shock protein (Hsp) 90 and 70 families, respectively. Hsp70 and Hsp90 can function independently, but their overlapping clientele list favors a collaboration of the two chaperones in processing specific clients.[7] The collaboration of Hsp90 and Hsp70 requires a functional and physical interaction of both chaperones using conserved residues across homologs.[7–10]

Grp94 has a similar architecture to other Hsp90s. It assembles as a homodimer with each monomer containing an N-terminal domain (NTD) that binds nucleotide, a middle domain (MD), and a C-terminal domain (CTD) for dimerization. Additional features of Grp94, such as an extended pre-N domain and relatively low ATP hydrolysis rates compared to other homologs, suggest mechanistic differences in functional activity.[11,12] ATP binding at the NTD drives large-scale conformational changes in Hsp90s that move the chaperone from the open state in which the CTDs are dimerized and the two NTDs are separated in a "V" conformation to a closed state with transient dimerization at the NTDs in addition to dimerization at the CTDs.[13] Like other Hsp70s, BiP is comprised of a nucleotide-binding domain (NBD) connected via an interdomain linker to the substrate-binding domain (SBD).[14,15] In the ATP-bound state, the NBD and SBD are docked, and the SBD has weak affinity for clients. Upon ATP hydrolysis, the domains separate once ATP is hydrolyzed to ADP to allow efficient trapping of clients in the SBD.[16,17]

The concerted actions of the cytosolic Hsp70 and Hsp90 in protein folding are regulated by coordinated cycles of ATP binding and hydrolysis by the two chaperones. Hsp70 initially binds clients in the ATP-bound state. This process is facilitated by J-domain containing co-chaperones (Hsp40s) that deliver clients to Hsp70 and stimulate ATP hydrolysis by Hsp70.[17] Once ATP is hydrolyzed, Hsp70 is converted to the ADP state, where it preferentially binds to the open apo form of Hsp90, delivering the client. In organisms and organelle lacking Hsp90 cochaperones, this is likely done through a direct interaction between Hsp70 and Hsp90.[10,18] In eukaryotes, the Hop/Sti1 cochaperone facilitates the Hsp70/Hsp90 interaction.[19–21] ATP binding and complete dimer closure of Hsp90 reduces the affinity for Hsp70 and leads to dissociation of Hsp70 from Hsp90.[22] Final client processing and release require ATP hydrolysis by Hsp90.[23] Hsp90 cochaperones facilitate Hsp90's progression through the chaperone cycle in eukaryotic organisms.[19,20] This nucleotide-regulated interaction of Hsp70 and Hsp90 supports the two chaperones' proposed sequential interaction with clients during folding.[24]

The mechanistic action of Hsp70 and Hsp90 is well studied in the bacterial and eukaryotic cytosolic proteins.[9,22,25,26] However, a detailed understanding of the synergistic collaboration of Grp94 and BiP in protein folding remains elusive. The folding of client proteins, including immunoglobulins, IGF, and thyroglobulin, require interactions with both Grp94 and BiP.[1,27] In immunoglobulin folding, successive interactions of BiP and Grp94 were proposed, where Grp94 favors interactions with mature forms of immunoglobulins and works downstream of BiP.[27] Grp94 was also shown to bind unfolded immunoglobulins as part of a large multi-chaperone complex, though evidence of a functional collaboration of BiP and Grp94 in folding is still lacking.[28] A direct interaction between BiP and Grp94 has been recently demonstrated, but the functional relevance of this interaction in protein folding is unknown.[18] A recent study focused on the effect of Grp94 and BiP on the folding of pro-IGF and reported that these proteins do not substantially contribute to the folding pathway of pro-IGF; however, this in vitro system lacked the essential co-chaperones that work in concert with BiP during client folding.[29] A key question in the potential collaboration of BiP and Grp94 in protein folding is the role of ATP binding and hydrolysis by Grp94 in client remodeling. This question arises from the intrinsic low ATPase activity and dimer closure rates of purified Grp94 compared to other Hsp90 homologs.[12,30,31] Additionally, Grp94 lacks the plethora of co-chaperones available to the cytosolic Hsp90s to regulate its ATPase cycle.[11]

Unlike Hsp70s, Hsp90s can also function in an ATP-independent manner. This ATP-independent role is important for stabilizing and preventing aggregation of model clients in vitro.[32–34] The stabilizing effect of Hsp90 may also serve important physiological roles. Hsp90 is important for stabilizing the client kinase V-src under heat stress conditions.[35] Interestingly, this chaperone activity required ATP binding but not hydrolysis by Hsp90. In contrast, both ATP binding and hydrolysis are required to chaperone V-src at lower temperatures. A similar phenomenon was observed for the cyanobacterial Hsp90, where ATP binding and hydrolysis were not required for refolding clients during heat stress.[36] ATP binding, but not hydrolysis is also sufficient for chaperoning of p53 protein by Hsp90 at physiological temperatures.[37] Together, this suggests that Hsp90 mechanisms may be client specific or adapt based on the environmental conditions. In this work, we sought to investigate the collaboration of Grp94 and BiP in protein refolding under heat-induced client misfolding and determine the requirements for chaperone activity by the two chaperones.

Results

The BiP chaperone system facilitates luciferase refolding

In the ER, BiP is a major player in the chaperoning of newly synthesized and misfolded proteins.[5] The interaction of BiP and its co-chaperones and how this may translate into client processing have been well studied.[38–40,15] The processing of clients by BiP is facilitated by J-proteins and nucleotide exchange factors (NEFs). In the ATP-bound state where BiP has high on/off rates with clients, J-proteins stimulate BiP's ATP hydrolysis and promote stable interactions with the client.[38] J-proteins may also recruit clients to BiP since some J-proteins interact with clients directly.[41,40] To ensure a complete ATPase cycle by BiP necessary for client folding, the NEFs Sil1 and Grp170 facilitate nucleotide exchange by BiP in the presence of J-proteins.[42] While client refolding by BiP and J-proteins in vitro has been shown, previous attempts to reconstitute client refolding by BiP, J-proteins, and NEFs were unsuccessful.[43]

To study the collaboration of Grp94 and BiP in protein refolding in vitro, we first sought to develop a protein reactivation assay for BiP that includes both a J-protein and a NEF. Towards this goal, we purified full-length human BiP along with a J-protein and the two NEFs of BiP, Sil1, and Grp170 (Figure S1(A)). Although Grp170 exists as a glycosylated protein in the ER, previous work has shown that bacterial derived Grp170 in absence of glycosylation does not abolish the NEF function in vitro.[44,45] We also confirmed the correct folding of our purified Grp170 construct using CD spectroscopy (Figure S1(B)). We chose the J-protein DnaJB11 (Erdj3) for this assay because it has been well characterized. DnaJB11 was shown to be particularly important in BiP folding pathways and was identified in a multimeric chaperone complex with both BiP and Grp94.[28,46] Similar to Hsp40 homologs, DnaJB11 is known to stimulate the ATP hydrolysis rates of BiP.[47]

We began by testing the activity of our purified proteins using an enzymatic coupled ATP hydrolysis activity assay. BiP alone had an ATPase activity of 0.01 nmol min^{-1}, and this activity was increased by ~13-fold when DnaJB11 was added (Figure 1(A)). Although lower fold enhancement rates by DnaJB11 have been reported, our results agree with a study that used a similar human BiP construct.[14] The NEF Sil1 alone also increased the BiP ATPase activity, which has been observed previously,[39,48] while Grp170 alone had no effect. The effect of Sil1 on the ATPase activity of BiP is attributed to its flexible N-terminus, which acts as a pseudo substrate and stimulates BiP activity similar to stimulation of BiP ATPase by BiP peptides.[39,49] In reactions containing BiP, DnaJB11, and Sil1, or BiP, DnaJB11, and Grp170, the BiP ATP hydrolysis rates were further increased by ~30 and ~38-fold for Sil1 and Grp170, respectively, suggesting a functional nucleotide exchange activity for our purified proteins. In the absence of BiP, other chaperone combinations did not yield appreciable ATPase activity.

Next, we tested the ability of an unfolded protein to stimulate BiP ATPase activity. We chose the ribosomal protein L2 as a model client since it is natively unfolded at low salt concentrations and it is known to bind to the *E. coli* Hsp70 and Hsp90 chaperones.[50] We confirmed the binding of BiP to His-tagged L2 using a protein–protein interaction assay (Figure S2). In ATPase assays, adding L2 alone increased the BiP ATPase activity by about 5-fold (Figure 1(B)). The addition of DnaJB11 to the mixture did not increase the ATPase hydrolysis rates beyond what was observed for BiP and DnaJB11 alone (Figure 1(B)). When the NEFs were subsequently added, Grp170 increased the ATPase rates by about 2-fold beyond what was observed in mixtures of BiP, DnaJB11, and Grp170 without L2. Interestingly, Sil1 did not further stimulate the ATPase rates in the presence of L2. These results suggest that Grp170 enhances the productive cycling of BiP both in the presence and absence of client, while BiP interactions with Sil1 do not result in an appreciable increase of BiP's conformational cycling in the presence of client.

After we demonstrated the stimulation of BiP by its co-chaperones, we set out to test the ability of the BiP system to refold a model client, firefly luciferase. Luciferase was heat denatured, and chaperone-dependent refolding was monitored in the presence of ATP. Luciferase alone did not spontaneously refold (Figure 1(C)). The addition of BiP alone was unable to refold luciferase, whereas the addition of BiP and DnaJB11 increased luciferase refolding by about 2.5-fold over spontaneous refolding. When BiP, DnaJB11, and Grp170 were present, the luciferase refolding rate was increased by about 3.8-fold over spontaneous refolding. In contrast to Grp170, Sil1 had a lesser effect on refolding rates of BiP, with a minimal enhancement of reactivation compared to reactions that do not include Sil1 (Figure 1(D)). This trend was apparent over a broad range of concentrations, with no enhanced refolding effect by Sil1 (Figure S3). Together, our results suggest that Grp170 commits BiP to productive folding pathways while Sil1 has a diminutive effect on folding by BiP.

Luciferase refolding requires ATP hydrolysis and peptide binding by BiP

Next, we tested the well-characterized BiP substitution mutants, the ATP hydrolysis defective (T229A) and peptide binding defective (V461F) mutants, in their ability to refold heat denatured luciferase.[51,52] The mutants were purified and shown to fold similarly to the wild-type (Figure S4 (A)). We went on to test the activity of the mutants

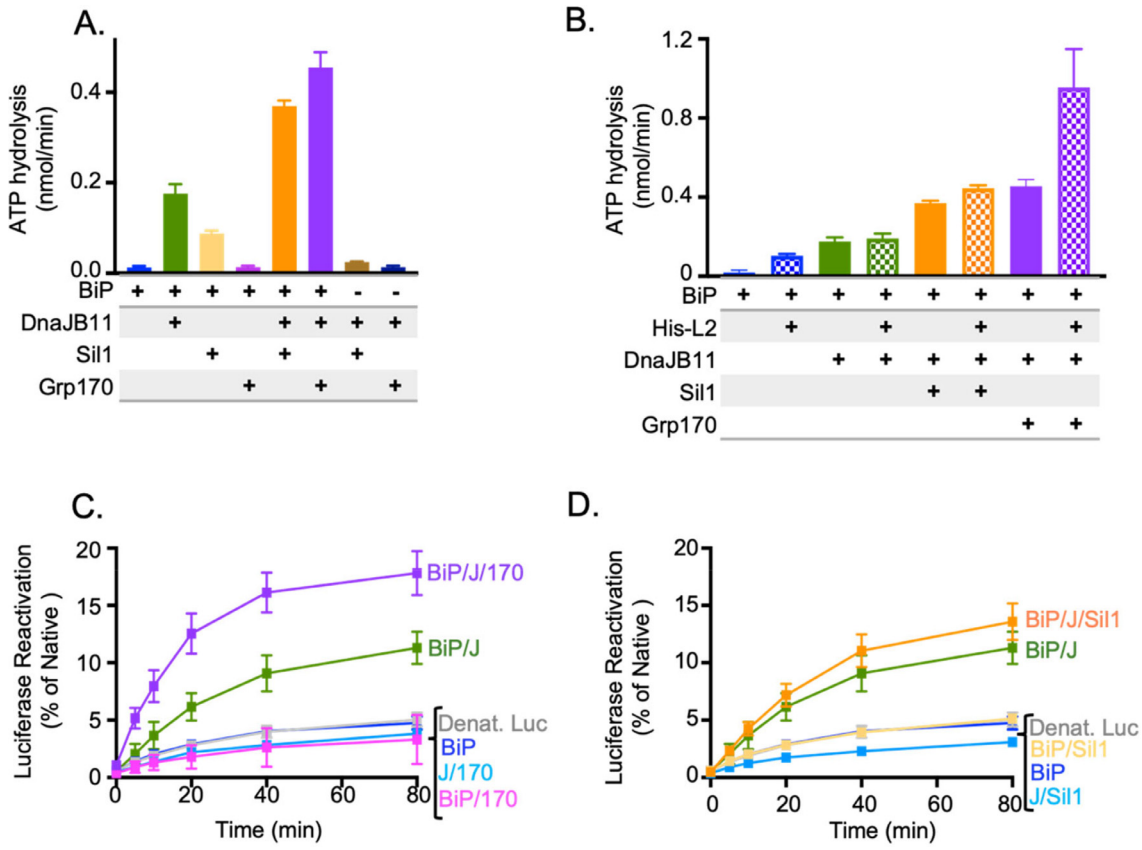

Figure 1. BiP and its co-chaperones reactivate luciferase denatured under milder conditions in vitro. (A) ATPase measurements were carried out using an enzyme coupled assay as described in *Methods*. Protein concentrations in all ATPase assays are 1 μM BiP, 1 μM DnaJB11 (J), 1 μM Sil1 and 0.1 μM Grp170 (170). (B) ATPase measurements were carried out as described in (A) with 1 μM BiP, 1 μM DnaJB11, 1 μM Sil1, 0.1 μM Grp170, and 1 μM L2. (C) Reactivation of 20 nM heat denatured luciferase by BiP, DnaJB11, and Grp170 as described in *Methods*. All luciferase reactivation assays include 2 μM BiP, 4 μM DnaJB11 (J) and 0.2 μM Grp170 (170). (D) Reactivation of 20 nM heat denatured luciferase by BiP, DnaJB11, and 0.4 μM Sil1. Data shown in A-D represent the average of at least three experiments ± sd.

in ATP hydrolysis assays. As expected, the BiP T229A mutant had a significantly reduced ATP hydrolysis activity even at higher concentrations, while BiP V461F showed higher basal activity compared to wild-type (WT) BiP (Figure S4(B)). Next, we tested the ability of the Hsp70 NR peptide, a 7-residue peptide (NRLLLTG) that binds to Hsp70s, to stimulate the BiP ATPase activity. We observed a peptide concentration-dependent increase in the BiP ATP hydrolysis activity with a ~7-fold increase in activity at the highest concentration tested (Figure S4(C)). The residual ATP hydrolysis of the BiP T229A mutant was also stimulated in the presence of increasing peptide concentrations. In contrast, the NR peptide had no stimulatory effect on the ATPase activity of the BiP V461F mutant even at high peptide concentrations, confirming the reduced ability of this mutant to engage the NR peptide.

When we tested the effect of co-chaperones on the ATP hydrolysis activity of the mutants, we found that the BiP T229A mutant had substantially reduced ATPase activity under all the conditions tested (Figure 2(A)). Interestingly, the V461F mutant was also defective in collaborating with co-chaperones. The stimulation of BiP V461F by DnaJB11 was reduced by 80% compared to WT. Consequently, the addition of Grp170 did not further increase the ATP hydrolysis activity of V461F with JB11, compared to BiP WT that demonstrated a 2.6-fold stimulation by Grp170. The defect in the collaboration of BiP V461F with DnaJB11 has been observed for other J-domain proteins and BiP V461F, and for the *E. coli* DnaK V436F mutant and DnaJ. In both cases, a defect in the direct interaction of the two proteins was observed, compared to WT protein.[53,54] Single point mutations in the substrate binding domain of Hsp70s have also been shown to affect interdomain coupling and co-chaperone interactions.[55] Additionally, we tested

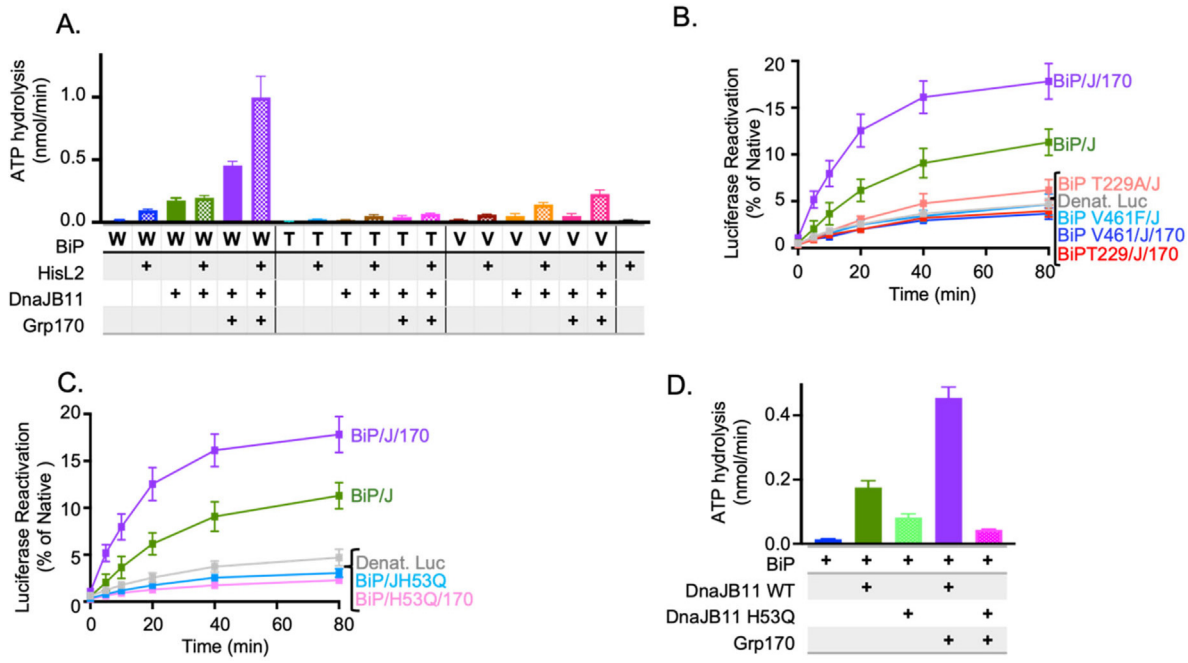

Figure 2. Client refolding requires BiP ATPase activity, peptide binding activity, and direct interaction with a J-protein. (A) ATPase measurements were carried out using an enzyme coupled assay as described in *Methods.* Protein concentrations are 1 μM BiP wild-type (W), T229A (T) or V461F (V) mutant, 1 μM DnaJB11, 0.1 μM Grp170, and 1 μM L2 (B) Reactivation of 20 nM heat denatured luciferase by DnaJB11 (J) and Grp170 (170) with wild-type (WT) BiP, BiP T229A, or BiP V461F. (C) Reactivation of 20 nM heat denatured luciferase by BiP and Grp170 with DnaJB11 WT or H53Q. (D) ATPase measurements were carried out with BiP, DnaJB11 WT or H53Q, and Grp170. Data shown in (A–D) represent the average of at least three experiments ± sd.

the effect of a larger client protein, L2, on the ATPase activity of BiP mutants. Partial stimulation by L2 was observed for BiP V461F, though reduced by ~50% compared to BiP WT. Similar reductions in stimulation were observed when DnaJB11 was added to V461F and L2. When all chaperone components were present with L2, the stimulation of BiP V461F was reduced by ~75% compared to WT. These results suggest that while the BiP V461F is unable to bind the NR peptide, it may retain partial ability to bind polypeptides or proteins, possibly due to additional interacting surfaces. Recent studies have identified a second peptide binding site in Hsp70s different from the canonical NR peptide binding site.[56] Our results with the BiP T229A mutant also suggest that the observed increase in ATP hydrolysis in the presence of L2 can be largely attributed to BiP and not the stimulation of Grp170's ATP hydrolysis activity. The BiP substitution mutants were then tested in luciferase refolding assays. As expected, the BiP T229A mutant completely failed to refold heat denatured luciferase (Figure 2(B)). Interestingly, the V461F mutant was completely ineffective in luciferase refolding, suggesting that the NR peptide binding region is important for client refolding (Figure 2 (B)). These results suggest that both ATP hydrolysis and peptide binding are required for the refolding activity of the BiP system in vitro, in agreement with previous studies with similar mutants in DnaK.[25]

J-proteins regulate substrate interactions with Hsp70s; some J-proteins assist in substrate capture and transfer substrates to Hsp70s through direct functional interaction.[57] A conserved histidine residue in the J-domain of J-proteins facilitates this interaction with Hsp70s. Substitution of this His residue to Gln decreases the Hsp70-J protein interaction. Lumenal DnaJB11 can bind unfolded substrates independent of the J-domain. Interactions between DnaJB11 and BiP release the client to BiP.[41,40] We purified and tested the corresponding substitution mutant in DnaJB11 (H53Q) in luciferase refolding assays. The H53Q mutant had similar structure and purity compared to the WT protein (Figure S4(D)). Incorporation of the DnaJB11 H53Q mutant completely abolished the ability of BiP to refold heat denatured luciferase (Figure 2 (C)). In ATPase assays using DnaJB11 H53Q, we observed residual stimulation of BiP ATPase activity compared to the wild-type, but the stimulation by the mutant was reduced by 50% compared to WT (Figure 2(D)). This DnaJB11-dependent stimulation of BiP ATPase activity was salt-dependent. At higher salt concentrations, the increase in activity by the WT protein was suppressed in comparison to stimulation observed in lower salt conditions,

while no stimulation was observed from the H53Q mutant (Figure S4(E)). Recent reports have shown electrostatic interactions between E. Coli DnaK and DnaJ, and may account for the salt dependence that is observed.[58] Interestingly, adding Grp170 did not further enhance the BiP ATPase activity and instead decreased the stimulation from the DnaJB11 mutant, suggesting that a functional BiP-DnaJB11 interaction is important for the subsequent activity of Grp170 (Figure 2(D)). Despite the ability of the DnaJB11 mutant to partially stimulate BiP ATPase activity, the mutant is likely unable to transfer bound substrates to BiP because of defective physical interactions with BiP.[40]

Grp94 collaborates with the BiP system to refold heat denatured luciferase under stronger denaturation conditions favoring aggregation

We sought to investigate the collaboration of the BiP system with Grp94 in the folding of heat denatured luciferase. In the bacterial and eukaryotic systems, initial refolding occurs on Hsp70, followed by a substrate transfer to Hsp90s for subsequent collaboration between the two chaperone systems to complete refolding.[25,22] In higher eukaryotes, the Hop/Sti1 co-chaperone facilitates the direct Hsp70-Hsp90 interaction and transfer of a substrate[59]; this co-chaperone is not present in bacteria, and the direct interaction between HtpG and DnaK allows for substrate transfer.[8] Since a Hop/Sti1 homolog has not been identified in the ER and a direct interaction has been observed for BiP and Grp94,[18] we reconstituted our chaperone-mediated folding system similar to the bacterial system. We added increasing concentrations of Grp94 to the BiP system. Under these conditions, we did not observe any increase in reactivation rates when Grp94 was added to the BiP system, even at relatively high concentrations (Figure S5(A)). Our data thus suggests that Grp94 could not collaborate with BiP, DnaJB11, and Grp170 in refolding heat denatured luciferase under the conditions tested.

In addition to acting in later stages during protein refolding, Hsp90s can also function as a 'holdase' to suppress the aggregation of substrate proteins and maintain them in a folding competent state for subsequent refolding.[60] When we added Grp94 to luciferase during heat denaturation under the milder denaturation conditions, we still did not observe much effect from Grp94 even in the presence of ATP (Figure 3(A)). To better mimic conditions of irreversible aggregation, we used stronger denaturation conditions by thermally denaturing high luciferase concentrations, a parameter that increases the kinetics of luciferase aggregation.[61] Luciferase at 160 nM and 320 nM was heat denatured and separated into soluble and insoluble fractions (Methods). The western blot profile shows an increase in insoluble luciferase aggregates with increasing luciferase concentrations as compared to luciferase from milder denaturing conditions at 20 nM (Figure S5(B)). When 160 nM of luciferase is heat denatured and then incubated with the BiP system, the ability of the BiP system to refold luciferase was significantly reduced (Figure 3(B)). The BiP system may only refold soluble luciferase and not the insoluble portion. Adding Grp94 directly to the chaperone mixture did not cause any significant increase in reactivation rates. Importantly, when Grp94 was incubated with luciferase during heat denaturation and the BiP system was subsequently added, there was an increase in reactivation by ~3-fold compared to the BiP system alone. When ATP was present with Grp94 and luciferase during the heating step, reactivation was increased by ~5 fold. Reactivation required BiP and DnaJB11 and was enhanced by 3-fold when Grp170 was present (Figure S5(C)). In contrast to Grp170, Sil1 did not support refolding of luciferase by the BiP system in collaboration with Grp94 (Figure S5(D)). We wanted to determine whether there was a threshold concentration of luciferase at which Grp94 could no longer facilitate productive refolding. We carried out luciferase reactivation assays at 320 and 800 nM luciferase and demonstrated that the effect of Grp94 on BiP refolding extends to even higher concentrations of luciferase. A greater fold increase with Grp94 and ATP is observed compared to the BiP system in all cases, with an ~22-fold increase at 800 nM luciferase and a ~5-fold increase at 160 nM (Figure 3(C)). These results thus point to a very significant role of Grp94 for protein refolding under stronger denaturation conditions in collaboration with BiP and its co-chaperones and a less important role under milder denaturation conditions.

We considered the possibility that the function of Grp94 is activated during the heat denaturation step to help the BiP system resolve some of the insoluble heat denatured luciferase during the subsequent refolding step. We reasoned that if Grp94 is activated during heating to disaggregate some of the insoluble luciferase fraction during refolding, then removing the insoluble fraction before refolding should reduce the observed effect from Grp94. Therefore, we designed an experiment where luciferase was heated in the presence or in the absence of Grp94. The insoluble fraction of luciferase was removed by centrifugation, and the soluble portion of luciferase was added into refolding buffer containing the BiP system. The amount of soluble luciferase refolded by spontaneous folding or by the BiP system alone was decreased compared to the total luciferase (Figure 3(D)). This is likely because some soluble luciferase became insoluble during the extra handling steps and was removed during centrifugation. In contrast, in the presence of Grp94, luciferase was refolded to a similar extent in both the total and soluble portions (Figure 3(D)). This result suggests that Grp94's primary

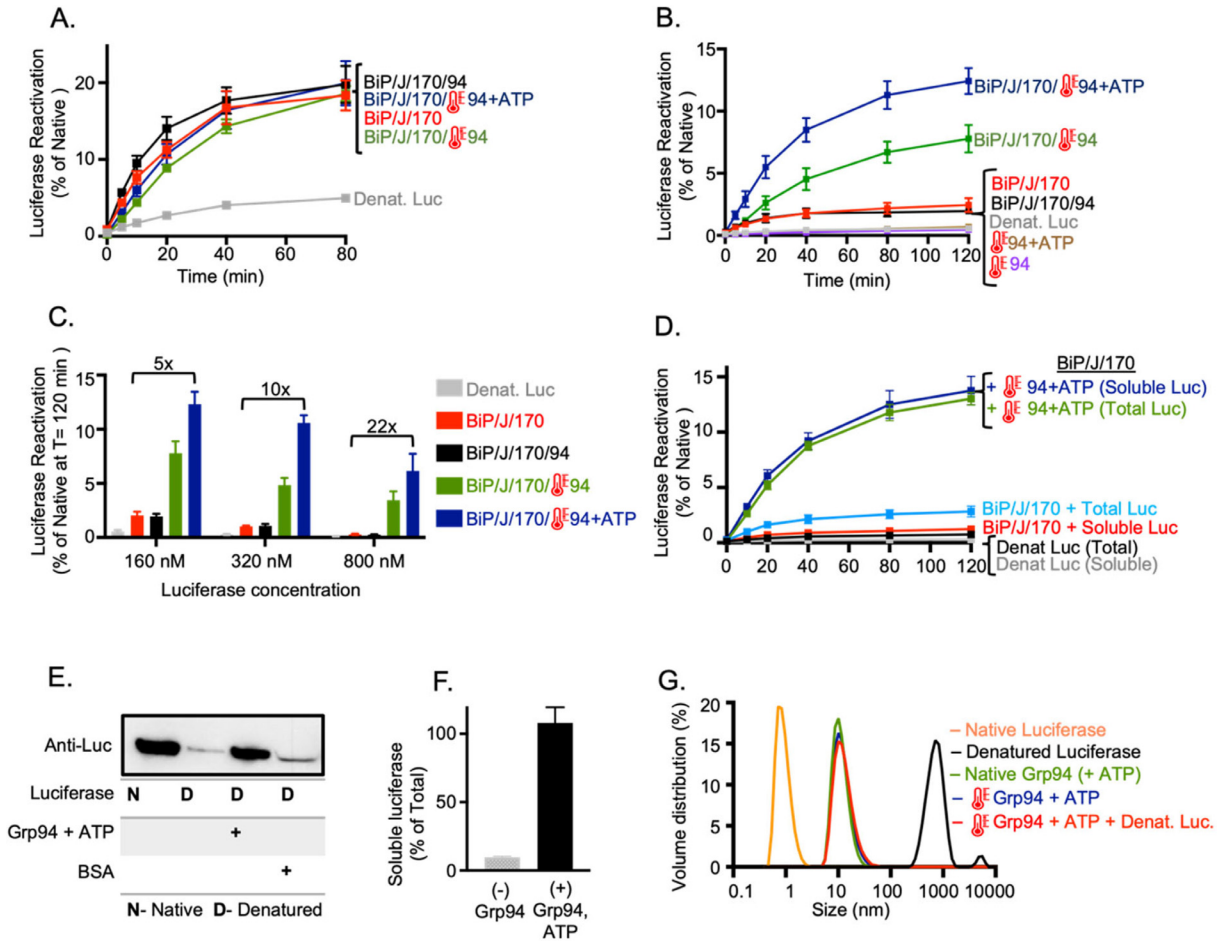

Figure 3. Grp94 collaborates with BiP to refold luciferase denatured under stronger denaturation conditions by acting as a holdase to prevent luciferase aggregation. (A) Reactivation of mildly denatured luciferase (20 nM) in the presence of 2 μM BiP, 4 μM DnaJB11 (J) 0.2 μM Grp170 (170) and 0.5 μM Grp94 (94). Where indicated by the thermometer, Grp94 with or without 0.5 mM ATP was present with luciferase during the heat denaturation step. Data shown represent the average of at least three experiments ± SEM (B) Luciferase (160 nM) was prepared as described in *Methods* in the presence or absence of 5 μM Grp94 with or without 5 mM ATP before diluting 10-fold into reaction mixtures. Where indicated, final chaperone concentrations are 2 μM BiP, 4 μM DnaJB11 (J) 0.2 μM Grp170 (170) and 0.5 μM Grp94 (94) (C) Maximal luciferase reactivation measured at the endpoint of the reaction (starting with 160, 320, or 800 nM during heat denaturation with Grp94 at 5, 10, and 25 μM, respectively) followed by dilution into reactions containing the BiP system (*Methods*). (D) Luciferase (160 nM) was heat denatured in the presence or absence of 5 μM Grp94 and 5 mM ATP as described in *Methods*. The total luciferase or the soluble fraction of luciferase was diluted 10-fold into buffer containing BiP, DnaJB11 (J), and Grp170 (170). Luciferase reactivation was measured over time. In (B-D), the data shown represent the average of at least three experiments ± sd. (E) Western blot of the soluble portion of luciferase prepared in the presence or absence of Grp94 and ATP. A representative image from at least three independent experiments is shown (F) Luciferase amounts from (E) were quantified by densitometry and expressed as a percentage of native luciferase. Data shown represent the average of at least three experiments ± SEM (G) Dynamic light scattering of heat denatured luciferase and Grp94, as described in *Methods*. Native luciferase and Grp94 are included as controls. The data shown are representative of at least four independent measurements.

chaperone activity occurs during the initial heat denaturation step by increasing the amount of soluble luciferase. To confirm this, we used our western blot analysis to determine the amount of luciferase in the supernatant and pellet fractions in the presence and absence of Grp94. Interestingly, the presence of Grp94 during heating completely restored the amount of luciferase in the soluble fractions (Figure 3(E and F)). This aggregation prevention activity of Grp94 was not dependent on ATP, and BSA could not substitute for Grp94 to prevent luciferase aggregation (Figure S6(A and B)).

We turned to dynamic light scattering to quantify the sizes of luciferase aggregates formed. Heat denatured luciferase formed very large

aggregated particles, with an approximate size of 825 nm (Figure 3(G)). Consistent with our western blot results, the addition of Grp94 resulted in a complete disappearance of these large luciferase aggregates, shifting the particle size to ~12 nm, which corresponds to the size of Grp94 alone. As a control, native luciferase did not aggregate and was observed around sizes of 1 nm. In agreement with the western blot quantification, ATP did not affect Grp94's ability to prevent aggregation as large aggregates were not observed when ATP was not present (Figure S6(C)). As a control, the heat incubation of Grp94 alone did not induce aggregation. Overall, our results suggest that under these conditions, Grp94 acts as a holdase to prevent luciferase aggregation during the heat denaturation step and maintains it in a "folding competent state" for subsequent refolding by the BiP system. The ATP-independent substrate binding activity of Grp94 agrees with previous studies of the human Hsp90 homolog.[32,34,60]

The ATPase activity of Grp94 is not required for collaboration with the BiP system in heat denatured luciferase refolding

In luciferase reactivation assays, the addition of ATP to Grp94 during the heat denaturation of luciferase significantly enhances the refolding reaction. Thus, we sought to investigate the role of ATP in the Grp94-mediated luciferase refolding reaction. Our attempts to measure ATP hydrolysis rates for full-length Grp94 were unsuccessful, with minimal ATP hydrolysis rates above the background observed. This is consistent with previous reports of a lack of a meaningful ATPase activity for full-length Grp94 derived from tissues or purified from bacteria.[12,31]

To determine if the small amount of ATP hydrolysis by Grp94 played a role in luciferase refolding, we introduced the previously characterized E103A and R448A ATP hydrolysis defective mutants of Grp94.[30] The E103A mutant binds ATP but cannot hydrolyze it, while the R448A mutant cannot populate the catalytically competent closed state required for ATP hydrolysis.[62] In addition, we introduced the D149N mutant, which is expected to have reduced ATP-binding activity.[63] We characterized the biophysical properties of these mutants using trypsin digests and circular dichroism spectroscopy (Figure S7(A and B)). The mutants behaved similarly to WT protein, suggesting the mutants had a similar secondary structure and overall fold. Additionally, we tested the substrate binding properties of the three mutants using an in vitro pull-down with His-L2, and all mutants had similar binding levels to the unfolded His-L2 (Figure S7(C)). When the mutants were tested for their ability to refold heat denatured luciferase, none of the mutants could refold luciferase when added directly to the BiP system (Figure 4 (A–C)). However, when the mutants were added to luciferase during heat denaturation, they all enhanced luciferase refolding by BiP, although to a lesser extent than the WT protein. The effect of ATP added during heat shock varied for each mutant. The E103A showed the most sensitivity to ATP, with a fold increase of ~2.7 over heat-shocked Grp94 only, compared to the ~1.6-fold increase observed for WT protein (Figure 4(A)). The enhanced sensitivity of the E103A may be due to a higher affinity for ATP, as has been observed for the human Hsp90β protein.[37] The addition of ATP enhanced the chaperone activity of the R448A mutant and restored refolding to similar levels observed for WT (Figure 4(B)). We expected less ATP-dependent activity from the D149N mutant, since this mutant is expected to be defective in ATP binding. However, the refolding activity of Grp94 D149N was still affected by ATP, though the overall refolding rate for this mutant was lower than WT protein (Figure 4(C)). The previously characterized ATP/Radicicol binding mutant of Grp94 involved mutations of both D149N and G153A.[63] The crystal structure of the Grp94 N-terminal domain in complex with nucleotide also shows interactions of both D149 and G153 with the bound nucleotide.[64] Thus, it is possible that the single D149N mutation of Grp94 still retains some residual level of nucleotide binding. Overall, our results suggest that ATP or nucleotide binding alone is enough to produce the enhanced effect from Grp94 and suggests that ATP hydrolysis may not be essential for the chaperone activity of Grp94 described here. The mitochondrial Hsp90, TRAP-1 also possess a similar ATP-dependent chaperone activity in collaboration with the mortalin (Hsp70) system, but it is unknown whether ATP binding alone is responsible for this effect.[65] In contrast to Grp94, a fully functional BiP system involving the DnaJB11 stimulated ATP hydrolysis and peptide-binding activity is required for luciferase refolding (Figure 4(D)).

A direct interaction of BiP and Grp94 is not required for heat denatured luciferase refolding

The observed ATP hydrolysis independent activity of Grp94 suggests a model where the effect of Grp94 is limited to its holdase activity and not involved in the subsequent refolding reaction. The functional collaboration between Hsp90 and Hsp70 in bacterial and eukaryotic cytosolic proteins requires a direct and physical interaction between the two proteins.[8,9,22] Thus, we tested for a functional role of Grp94 in the subsequent refolding step using a previously characterized Grp94 mutant with reduced binding to BiP.[18] Grp94 K467A had similar biophysical properties and substrate binding activity to wild-type Grp94 (Figure S7(A–C)). We confirmed the decreased binding of Grp94 K467A to BiP using a protein–protein interaction assay with biotinylated Grp94

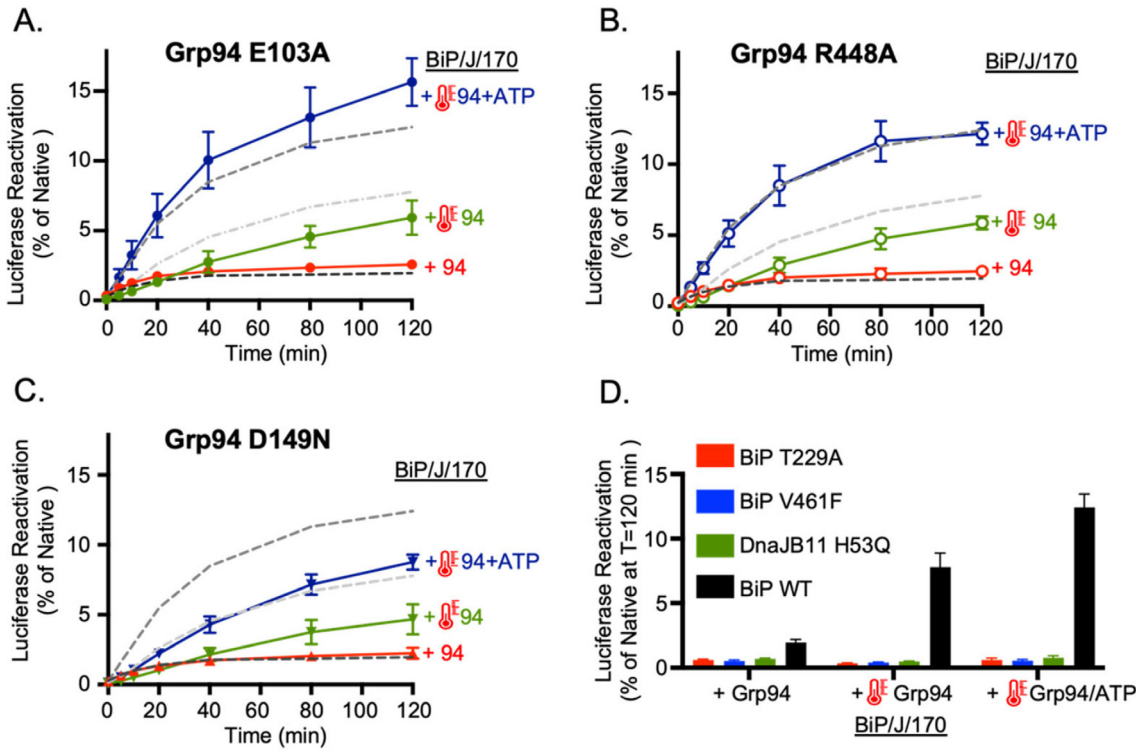

Figure 4. ATP hydrolysis by Grp94 is not required for collaboration with BiP in luciferase refolding. Luciferase reactivation using (A) Grp94 E103A, (B) Grp94 R448A, or (C) Grp94 D149N. (A-C) Luciferase refolding by BiP, DnaJB11 (J), Grp170 (170), and Grp94 ATP defective mutants. The red thermometers indicate Grp94/ATP was heat denatured with luciferase (*Methods*). For comparison, luciferase reactivation with WT Grp94 as untreated (black dashed line), heat incubated (light grey dashed line), and heat incubated with ATP (dark grey dashed line) are indicated. (D) Reactivation of luciferase at 120 minutes with BiP (T229A, V461F, or WT), DnaJB11 (WT or H53Q), Grp170, and Grp94. In (A-D), the data shown represent the average of at least three experiments ± sd.

labeled at primary amines (Figure S8). We performed our protein interaction assays in the presence of ADP based on previous reports that show that the Grp94 binding region on BiP only becomes available when BiP is in the ADP conformation.[18] Consistent with previous results, full-length Grp94 bound BiP robustly under ADP conditions while BiP binding to Grp94 K467A was significantly reduced. As a control, we tested binding of the Grp94 E103A mutant, which bound BiP similar to wild-type Grp94, suggesting that the observed defect in Grp94 K467A binding was not simply due to the introduction of a mutation. When Grp94 K467A was tested in luciferase refolding, it could refold luciferase similar to wild-type levels (Figure 5(A)). This suggests that the physical interaction of BiP and Grp94 was not necessary for luciferase refolding under these conditions.

For further confirmation of the lack of direct BiP-Grp94 interaction, we asked if the presence of substrate can stabilize a ternary complex formation of BiP and Grp94 under ATP conditions. Biotinylated Grp94 alone or in a complex with luciferase was diluted into refolding buffer with different chaperone combinations. Similar experiments with chaperones in the absence of luciferase were performed in parallel. In the presence of ATP, there was inefficient binding of BiP to Grp94, and the presence of luciferase did not improve BiP binding (Figure 5(B)). The addition of DnaJB11 significantly improved BiP binding to Grp94, though the binding levels were still low compared to that observed under ADP conditions (Figure S8 WT and quantified in Figure 5(B-C) as BiP(+ADP)). Similar to our results, the bacterial and yeast J-proteins also stabilize an Hsp70-Hsp90 complex under ATP conditions.[10] The effect of DnaJB11 on the stabilization of a Grp94-BiP complex was not dependent on substrate, since similar amounts of BiP were bound in the absence of luciferase. In the presence of Grp170, much less BiP bound to Grp94 with or without luciferase (Figure 5(B)). This is consistent with the ability of Grp170 to stimulate nucleotide exchange by BiP and allow ATP to re-enter, thus, resetting the cycle. The proposed substrate transfer from Hsp70 to Hsp90 requires ATP hydrolysis by Hsp90 and direct interaction between the chaperones.[8,22] To investigate if a similar substrate transfer mechanism occurs in our refolding assays, we tested the formation of the Grp94-BiP complex using the E103A and K467A mutants of Grp94,

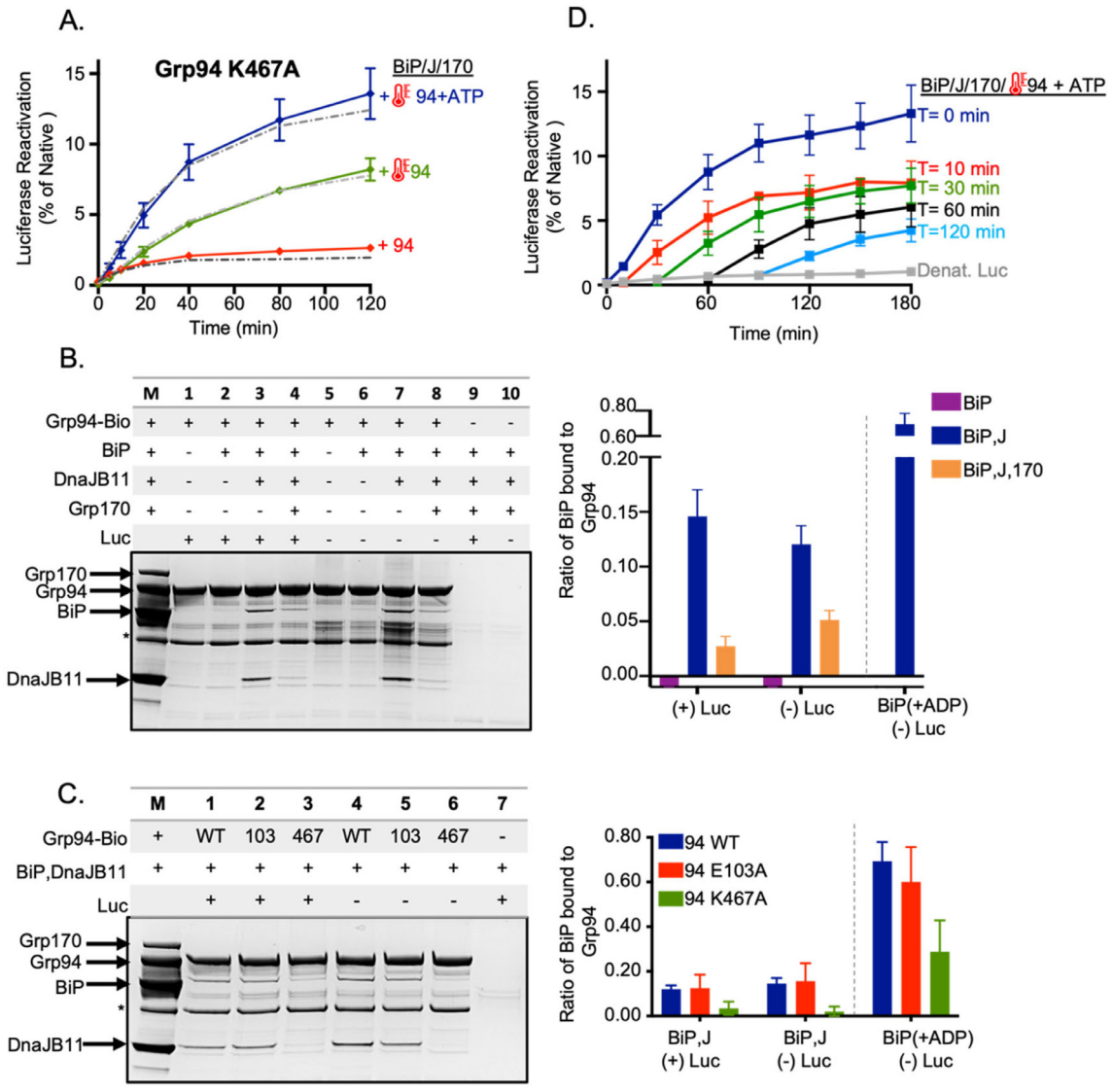

Figure 5. A physical interaction between BiP and Grp94 is not required for luciferase refolding. (A) Luciferase refolding in the presence of BiP, DnaJB11 (J), Grp170 (170), and Grp94 K467A. Thermometers indicate Grp94/ATP was heat denatured with luciferase. For comparison, luciferase reactivation with WT Grp94 as untreated (black dashed line), heat incubated (light grey dashed line), and heat incubated with ATP (dark grey dashed line) are indicated. Data shown represent the average of at least three experiments ± sd. (B) Interaction of Grp94-biotin and BiP was determined using a streptavidin pull-down assay as described in *Methods*. Proteins bound to Grp94-biotin were determined by SDS-PAGE. M indicates markers for Grp170, Grp94, BiP, and DnaJB11. (*) indicates a degradation product of Grp94 confirmed by mass spectrometry. A representative gel from at least three independent experiments is shown. The gels from all experiments were quantified to show the amount of BiP bound to Grp94-biotin for comparison. Quantification of BiP bound to WT Grp94-biotin under ADP conditions in absence of luciferase (Figure S8) is included in the bar graph. The mean ± SEM is indicated in the bar graph on the right. (C) Streptavidin pull-down assays were performed as described in (B) with biotinylated Grp94 WT or mutant, as indicated, with BiP and DnaJB11. A representative gel from at least three independent experiments is shown. The gels from all experiments were quantified to show the amount of BiP bound to Grp94-biotin. The mean ± SEM is indicated in the bar graph on the right. For comparison, the quantification of BiP bound to WT or mutant Grp94-biotin under ADP conditions in the absence of luciferase (Figure S8) is included in the bar graph. (D) Luciferase reactivation with delayed addition of chaperone components to denatured luciferase. Luciferase was heat denatured with Grp94/ATP and then preincubated at 30 °C for the indicated time. Luciferase/Grp94 was then diluted into chaperone mixtures containing BiP, DnaJB11, and Grp170, and luciferase reactivation was monitored (*Methods)*. Data from at least three measurements are shown as mean ± sd.

which are defective in ATP hydrolysis and BiP binding, respectively. The E103A mutant bound to BiP similarly to wild-type, while the K467A mutant had significantly less binding (Figure 5(C)). Together, our results suggest that under ATP conditions, DnaJB11 converts a fraction of BiP molecules to the ADP conformation required for interaction with Grp94. Since the presence of luciferase did not contribute to the stabilization of this complex, we propose that a direct substrate transfer from Grp94 to BiP does not occur under these conditions. This also explains why the ATP hydrolysis defective and BiP-binding defective mutants of Grp94 were functional in luciferase refolding.

In light of these results, we questioned how substrates are made available to the BiP system for refolding after heat incubation. We performed delayed addition experiments where heat denatured luciferase was pre-incubated at 30 °C for extended periods before adding BiP and co-chaperones. We hypothesized that if luciferase is released into solution and picked up by the BiP system, the amount of refoldable luciferase will decrease over time due to the trapping of luciferase folding intermediates that are not retrievable by the BiP system. We indeed observed that after extended incubation, the amount of luciferase refolded was significantly decreased (Figure 5(D)). The presence of BiP and DnaJB11 during the incubation period improved the refolding yield, while other chaperone combinations were less effective at improving the refolding (Figure S9(A)). Additionally, BSA could not effectively replace Grp94 in these refolding experiments (Figure S9(B)). These results also point to a model where substrate release or accessibility may serve as a rate-limiting step in the Grp94-mediated refolding reaction. This is supported by our observation that while Grp94 completely restored the amount of soluble luciferase under stronger denaturation conditions favoring aggregation, the BiP system could only refold ~15% of soluble luciferase (Figure 3(B, E–G)). When we probed the localization of luciferase on Grp94 during the time course of refolding, we found that a significant amount of luciferase remained bound even after 120 mins of refolding (Figure S10). We note that our results do not exclude the possibility of folding of luciferase while bound to Grp94. If this were the case, however, we would expect that a Grp94-BiP complex would enhance refolding rates by increasing recruitment of BiP to luciferase bound to Grp94, but our results with the K467A mutant argue against this possibility.

The ATPase activity of bacterial Hsp90 is required for luciferase refolding in collaboration with the DnaK system

Finally, we asked if a similar ATP hydrolysis independent activity observed for Grp94 occurs in the *E. coli* Hsp90 (HtpG) system. Luciferase refolding experiments were performed similarly under stronger denaturation conditions using high luciferase concentrations. To observe an HtpG effect, we used higher DnaJ concentrations where luciferase refolding rates by the KJE system alone are minimal.[66] At these high DnaJ concentrations, adding HtpG directly to KJE improved refolding ~3-fold (Figure 6(A)). When HtpG was added during heat shock, we observed no further improvement in refolding yields. The addition of ATP during heat shock caused a slight improvement over the course of the refolding reaction but to a lesser extent compared to the effect observed with Grp94. Next, we tested the ability of the ATP binding (D80N) and ATP hydrolysis (E34A) defective mutants of HtpG to support luciferase refolding with KJE. Interestingly, both mutants were completely inactive in luciferase refolding even when added during heat shock (Figure 6(B)). This suggests a stricter requirement of the ATPase activity of HtpG compared to Grp94 in luciferase refolding.

Discussion

In this study, we have shown the collaboration of BiP and its co-chaperones in refolding heat denatured luciferase in an ATP-dependent manner. This relatively simple assay can be exploited in future studies with ER chaperones. In the ER, BiP has 8 J-proteins with different functions, usually categorized as facilitators of folding or facilitators of degradation. Our results confirm that DnaJB11 facilitates the folding of model client proteins by stimulating BiP's ATPase activity and enhancing BiP's chaperone activity.[46,47,67] The NEF Sil1 was less effective in assisting BiP chaperone activity than Grp170 in these studies, as observed by the lack of BiP ATPase stimulation in the presence of a client and the lack of enhancement in chaperone function. Grp170 and Sil1 have different binding modes with BiP and utilize distinct mechanisms to accelerate nucleotide release and stimulate substrate release from BiP.[68] The unstructured N-terminus of the Sil1 homologs, Fes1 and HspBP1, interacts with the peptide binding region of Hsp70. This interaction facilitates substrate release and blocks rebinding with substrate.[69,70] Fes1, has been recently shown to be involved in degradative pathways by ensuring the release of persistent misfolded proteins from Hsp70 for targeting to proteasomal degradation pathways.[70,71] In other studies, N-domain derived peptides of Sil1 were shown to bind to BiP and effectively compete with BiP clients; thus, Sil1 may serve a similar pro-degradation role in the ER.[39] This may explain why the enhanced chaperone activity of BiP (as shown for BiP, DnaJB11, and Grp170 in the presence of client) is not observed with Sil1. The role of Grp170 in enhancing BiP's chaperone activity is consistent with the significant role of its cytosolic homolog Hsp110 in refolding pathways of Hsp70

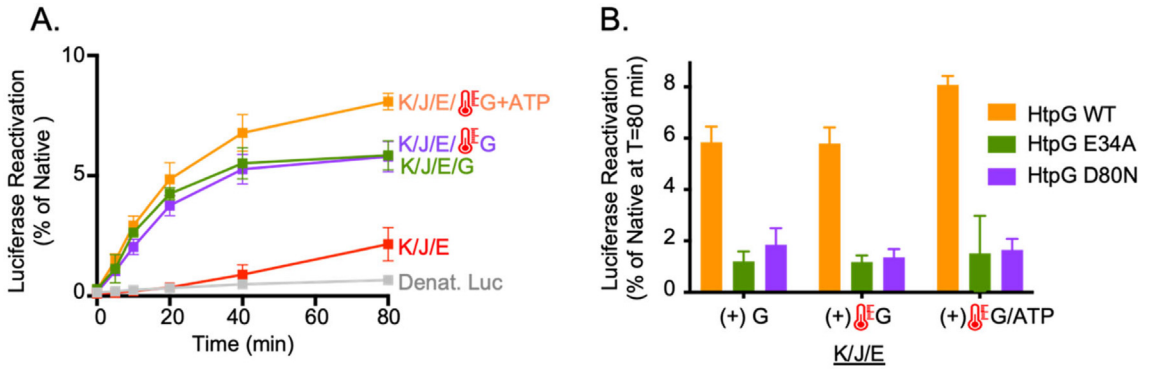

Figure 6. The collaboration of bacterial HtpG and DnaK requires the ATPase activity of HtpG. (A) Luciferase was heat denatured under stronger denaturation conditions that favor aggregation as described in *Methods.* Where indicated by a thermometer, HtpG/ATP was present during denaturation. Luciferase/HtpG was diluted into reaction buffer containing 0.95 μM DnaK (K), 0.7 μM DnaJ (J), and 0.065 μM GrpE (E), and reactivation was measured as a function of time. HtpG (G) was present at a final concentration of 0.5 μM. (B) Luciferase reactivation was carried out as in (A) with WT or mutant HtpG. In (A-B), data from at least three measurements are shown as mean ± sd.

and supports a general mechanistic action for this family type.[72,73]

We have also shown the collaboration of Grp94 and BiP in protein remodeling. We propose a model where the BiP system alone can refold misfolded substrates formed under milder denaturation conditions. Grp94 does not play a major role under milder denaturation conditions. However, under stronger denaturation conditions where the aggregation kinetics are increased, Grp94 offers a protective role by suppressing substrate aggregation and maintaining it in a state amenable to refolding (Figure 7). Upon stress removal, the substrate is released into solution or held in a state accessible by BiP and its co-chaperones for downstream processing in a Grp94-independent manner.

The observed lack of ATP-hydrolysis dependent chaperone activity of Grp94 is consistent with its

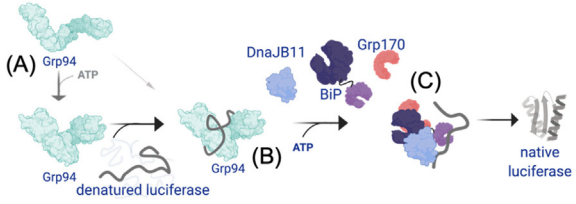

Figure 7. Model showing the collaboration of Grp94 and BiP in protein remodeling under heat shock conditions. (A) Grp94 in the apo or ATP bound 'open' state binds to denatured luciferase during heat shock to prevent protein aggregation. (B) When stress is removed, Grp94 releases substrate into the solution, which is then picked up by the BiP system. Substrate release by Grp94 is enhanced by ATP binding (C) BiP and its co-chaperones refold denatured luciferase back into the native state. Model was created using Biorender.com.

low dimer closure and ATP hydrolysis rates at temperatures below 37 °C.[12] In vivo, the ATPase activity of Grp94 is required for folding of Toll-like receptors (TLRs) and insulin-like growth factor II (IGF-II).[74,75] Thus, although we did not observe an ATP-dependent chaperone activity of Grp94 under our in vitro conditions, it is plausible that certain factors may serve to enhance Grp94 chaperone activity in vivo. So far, only two co-chaperones have been identified for Grp94, MZB1 and CNPY3.[76,77] However, they appear to be substrate protein specific instead of general co-chaperones. Both associate with Grp94 in a nucleotide-dependent manner but do not affect Grp94's overall ATPase activity.

Our results also point to an essential role of Grp94 in protecting proteins from aggregation during heat stress. A key finding in this study is the sensitivity of Grp94 to ATP added during the heat denaturation. The mitochondrial Hsp90 is similarly activated by ATP.[65] In the case of Grp94, we show that nucleotide binding, but not hydrolysis, is responsible for this increased activity. In vivo, ATP binding but not hydrolysis is sufficient for the folding of integrins by Grp94.[75] ATP binding does not affect the on-rate of substrate binding by Grp94 but affects the subsequent refolding reaction by increasing overall reaction rates. Since Grp94 does not seem to be directly involved in the actual refolding reaction, we reason that ATP binding leads to a conformational change in Grp94 that allows for more transient substrate binding or interactions such that substrate is more accessible by the BiP system for refolding. The role of ATP in promoting dynamic interactions of Hsp90 has been demonstrated for the cytosolic Hsp90β protein. ATP binding causes structural rearrangements in Hsp90 that may lead to dissociation of the chaperone-client complex.[37] ATP binding has also been shown to induce a conformational change in Grp94, wherein the two NTDs dimerize to form a semi-closed structure distinct

from the catalytically competent closed state.[64] How the ATP-induced conformational changes in Grp94 are coupled to client interactions and/or processing remains elusive.

We also observed that the Grp94-luciferase complex was very stable, and substrate availability to the BiP system was still rate-limiting. This may be a physiological role of Grp94, as previous in vivo research has indicated that Grp94 forms very stable complexes with integrins and TLRs, facilitating their transport to the cell surface.[75] Additionally, Grp94 present at the cell surface provides a scaffolding surface for binding and dimerization of HER2 in breast cancer cell lines with HER2 overexpression.[78] Disrupting the Grp94-client interaction with inhibitors or monoclonal antibodies inhibits trafficking and enhances the degradation of these clients. This suggests that stable chaperone-client interactions may be a hallmark of Grp94 that is necessary to promote the assembly and transport of client proteins.

Finally, we show that BiP cannot promote a retrograde transfer of the substrate from Grp94 under these conditions and that the direct interaction with Grp94 is not essential for substrate capture. This is consistent with previous results showing that individual N- and C-domains of cytoplasmic Hsp90 can independently protect substrates from aggregation and present them to Hsp70 for subsequent refolding.[79] Since most of the Hsp70-Hsp90 interaction sites are located in the M-domain, the lacking requirement of the M-domain suggests that direct interaction of the two chaperones is not required for refolding.[8,9] Instead of a direct retrograde transfer from Hsp90 to Hsp70, the co-chaperone Tpr2 and the 20S proteasome activator PA28 promote substrate release from Hsp90 and capture by Hsp70.[80,81] These proteins thus play similar roles to Hop/Stil in the forward reaction of substrate transfer from Hsp70 to Hsp90.[59] To date, no Hop/Sti1 homolog has been found for Grp94, and it remains to be determined if such a substrate release factor for Grp94 is present in the ER.

Materials and Methods

Plasmids and proteins

All human ER chaperone constructs were synthesized as fusion proteins with an N-terminal His_6 sumo tag (GenScript) with a TEV cleavage site. The fusion constructs were cloned into pET15b at the Nde1/BamHI restriction sites. The proteins were overexpressed in BL21 (DE3) cells (Grp94, BiP, Sil1), Origami cells (DnaJB11), or Rosetta2(DE3) pLysS cells (Grp170). Cells were grown at 37 °C until the OD_{600} reached 0.6–0.8 and induced with isopropyl β-d-1-thiogalactopyranoside (IPTG). The harvested cells were lysed using a French Press and separated by affinity chromatography using a HisTrap column. Removal of the His-sumo tag was carried out through proteolysis with the TEV protease. Proteins were further purified by affinity chromatography using a HisTrap column (Cytiva), ion-exchange with a MonoQ column (Cytiva), and size-exclusion using a Superdex 200 column (Cytiva) as described in *SI Methods*. HtpG WT and mutant,[25] DnaK,[82] DnaJ,[82] His-L2,[50] TEV protease[83] were prepared as described.

All mutations in pET15-Grp94, pET15-BiP and pet15-DnaJB11 were constructed using site-directed mutagenesis with the QuiKchange Lightning kit (Agilent). All mutations were verified by DNA sequencing.

ATP hydrolysis assays

ATP hydrolysis assays[84] were performed as previously described with modifications. Steady-state ATP hydrolysis was measured at 37 °C using a pyruvate kinase/lactate dehydrogenase (PK/LDH) enzyme-coupled assay. Buffer conditions were: 25 mM Hepes 7.5, 50 mM KCl, 2 mM DTT, 5 mM $MgCl_2$, 2 mM ATP, 1 mM phosphoenolpyruvate, 250 μM NADH, and PK/LDH (Sigma: 100-fold diluted). Protein concentrations were 1 μM BiP, 1 μM DnaJB11, 1 μM Sil1 and 0.1 μM Grp170. BiP, DnaJB11, Sil1, and Grp170 concentrations are for monomeric protein.

Luciferase reactivation assays

Luciferase reactivation assays[9] were performed as previously described with modifications. For denaturation at milder conditions, 20 nM Firefly Luciferase (Promega) was heat denatured at 45 °C for 7 mins, followed by a 1 min incubation at 4 °C. Denatured Luciferase (55 μL) was transferred to renaturation buffer (20 μL) containing 25 mM Hepes-KOH 7.5, 50 mM KCl, 0.1 mM EDTA, 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, 50 μg/mL bovine serum albumin (BSA), 2 mM ATP, an ATP regenerating system (20 mM phosphocreatine, 60 μg/mL Creatine kinase) with or without chaperones at the indicated concentrations. For stronger denaturation conditions, 160, 320, or 800 nM luciferase was heated at 45 °C for 7 mins, cooled at 4 °C for 5 mins, and diluted 10, 20, or 50-fold respectively into renaturation buffer containing 2 μM BiP, 4 μM DnaJB11 and 0.2 μM Grp170. ATP at a final concentration of 0.5 mM was added to all samples to correct for signal intensity. Luciferase activity was determined by transferring 5 μL of samples to 120 μL of Luciferin solution (25 mM Tris-HCl 7.8, 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 50 μg/mL Luciferin), and luminescence was measured using a plate reader (BioTek Cytation 5). BiP, DnaJB11, Sil1, and Grp170 concentrations are calculated as monomeric proteins. Grp94 and BSA are calculated as dimeric proteins.

Western blotting

Heat denatured luciferase was prepared as described and centrifuged at 17,200 rcf for 5 min at 4 °C. Aliquots from the supernatant were boiled in NuPAGE LDS Buffer and separated on a 4–20% Bis-Tris gel (GenScript) in the presence of SDS. The gel was transferred to a nitrocellulose membrane. The blot was incubated with 1 μg/mL of Goat Anti-Luciferase IgG (Promega) for 1 hour and 0.1 μg/mL of Donkey Anti-Goat IgG conjugated with horseradish peroxidase (Promega) for 1 hour and was visualized with a chemiluminescent detection kit (ThermoFisher Scientific) using a ChemiDoc Imager (Biorad). Images were processed with the ImageJ software (http://imagej.nih.gov).

Dynamic Light Scattering (DLS)

Dynamic light scattering measurements were performed on Zetasizer Nano S (Malvern Instruments). Heat denatured luciferase or Grp94 individually or together were equilibrated to 25 °C in measurement buffer (25 mM Hepes-KOH 7.5, 50 mM KCl, 0.1 mM EDTA, 10 mM $MgCl_2$, 0.1 mM $CaCl_2$) prior to measurement. At least four independent experiments were carried out per sample, and each measurement is an average of ~12 runs (measurements were performed twice). The buffer viscosity of 0.8401cP was used for calculations.

Trypsin digests

Grp94 (20 μg) WT or mutant was digested with trypsin (20 ng) at 37 °C for 0, 5, 10, and 30 min in 25 mM Hepes-KOH, 50 mM KCl and 2 mM DTT. 10 μL aliquots were removed at the indicated times, boiled in NuPAGE LDS Buffer, and run on a 4–12% Bis-Tris Gel (ThermoFisher Scientific).

Circular Dichroism (CD) spectroscopy

CD spectrometry was performed on an AVIV Model 435 Circular Dichroism Spectrometer. Samples of Grp94 were analyzed in a 1 mm path length quartz cuvette at 25 °C, and spectra were collected from 260-200 nm. Data points were collected every 1 nm with a 3 second averaging time. Grp94 WT or mutant, BiP WT or mutant and Grp170 was buffer exchanged into CD buffer (10 mM Tris–HCl, 100 mM KCl, 5% glycerol, and 0.2 mM TCEP) at a final concentration of 2 μM for Grp94 and BiP, and 0.5 μM for Grp170. DnaJB11 WT or mutant was diluted to 2 μM in CD buffer. Samples were run in triplicate.

Protein-protein interaction assays

Ni-NTA protein protein interaction assay. Interaction of BiP or Grp94 with L2 was measured using a protein–protein interaction assay. Protein solutions containing 2 μM wild-type (WT) or mutant protein and 4 μM His-L2 were incubated for 10 min at room temperature in reaction mixtures (50 μL) containing binding buffer (25 mM Hepes-KOH (pH 7.5), 50 mM KCl, 10% glycerol (vol/vol), 0.01% Triton X-100 (vol/vol), and 2 mM DTT). The samples were added to 50 μL Ni-NTA Agarose (1:1 slurry, ThermoFisher Scientific) and incubated for 5 min with mixing before dilution with 0.4 mL wash buffer (25 mM Hepes-KOH (pH 7.5), 50 mM KCl, 25 mM imidazole, and 0.01% Triton X-100 (vol/vol)). Samples were transferred to fresh tubes to minimize nonspecific binding and centrifuged for 1 min at 1000*g* at 4 °C. The recovered beads were incubated twice with 0.4 mL wash buffer with mixing, and bound proteins were eluted with buffer containing 75 mM KCl and 250 mM imidazole. Samples were boiled in NuPAGE LDS Buffer and run on a 4–12% Bis-Tris gel (GenScript).

NeutrAvidin/streptavidin protein protein interaction assays. Interaction between biotinylated Grp94 and BiP was measured using a protein–protein interaction assay. Protein solutions containing 0.5 μM wild-type or mutant Grp94 and 2 μM BiP-Bio were incubated in 2 mM ADP and 10 mM $MgCl_2$ for 30 min at 30 °C in reaction mixtures (50 μL) containing binding buffer (25 mM Hepes-KOH 7.5, 50 mM KCl, 0.1 mM EDTA, 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, 50 μg/mL bovine serum albumin (BSA-Sigma-Aldrich)). The reaction mixtures were diluted with 0.25 mL wash buffer (50 mM Tris 7.4, 75 mM KCl, 0.1% Tween 20) and added to 25 μL dry Streptavidin/NeutrAvidin Magnetic Speedbeads (Cytiva). The samples were incubated for 10 min at room temperature with mixing, and beads were captured using DynaMag 2 (Thermo Fisher). The recovered beads were washed 3x with 0.5 mL binding buffer, and bound proteins were eluted with NuPAGE LDS Buffer and run on a 4–12% Bis-Tris gel (Genscript). Interaction of Grp94 with BiP under ATP conditions was performed using 2 mM ATP and an ATP regenerating system instead of ADP.

Sample preparation for mass spectrometry sequencing

The protein sample (5 μM) was boiled in NuPAGE LDS Buffer and separated on a 10% Tris-glycine gel using SDS-PAGE. The gel was incubated with a fixing solution (50% methanol and 10% glacial acetic acid) for 25–30 min. The gel was stained, and the band was removed and stored in 5% acetic acid. The sample was sent to the CCIC Mass Spectrometry and Proteomics Facility at Ohio State University (OSU) for analysis.

CRediT authorship contribution statement

Yaa S. Amankwah: Supervision, Formal analysis, Investigation, Conceptualization, Methodology, Visualization, Writing - original draft, Writing - review & editing. **Preston Collins:** Formal analysis, Investigation, Visualization. **Yasmeen Fleifil:** Formal analysis, Investigation, Visualization, Writing - original draft, Writing - review & editing. **Erin Unruh:** Formal analysis, Investigation, Visualization, Writing - original draft, Writing - review & editing. **Kevin J. Ruiz Márquez:** Formal analysis, Investigation. **Katherine Vitou:** Formal analysis, Investigation, Visualization. **Andrea N. Kravats:** Funding acquisition, Supervision, Formal analysis, Conceptualization, Methodology, Visualization, Writing - original draft, Writing - review & editing.

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Competing Interest Statement

The authors declare no competing interest.

Appendix A. Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.jmb.2022.167762.

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molecular chaperones

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Abbreviations:
Grp94, Glucose regulated protein 94; BiP, Binding immunoglobulin protein; Grp170, Glucose regulated protein 170

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