The Chaperoning Properties of Mouse Grp170, a Member of the Third Family of Hsp70 Related Proteins†

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ABSTRACT: The 170 kDa glucose-regulated protein (grp170) is an endoplasmic reticulum resident protein that shares some sequence homology with both the hsp70 and hsp110 heat shock protein (hsp) families, yet is representative of a third and unique family of stress proteins. Despite observations indicating important roles in normal cellular functions, the in vitro chaperone properties of grp170 have not been rigorously examined. We have cloned mouse grp170 and expressed the recombinant protein in a baculovirus expression system. The function of recombinant grp170 was then assessed by determining its ability to bind to and prevent aggregation of heat-denatured luciferase. Grp170 maintains heat-denatured luciferase in a soluble state in the absence of ATP. In the presence of rabbit reticulocyte lysate, grp170 can refold and partially restore function to denatured luciferase. The chaperoning function of grp170 was also studied using domain deletion mutants, designed using the crystal structure of DnaK and the theoretical secondary structure of hsp110 as guides. Unlike hsp70 and hsp110, grp170 appears to have two domains capable of binding denatured luciferase and inhibiting its heat-induced aggregation. The two domains were identified as being similar to the classical β-sandwich peptide binding domain and the C-terminal α-helical domain in hsp70 and hsp110. The ability of the C-terminal region to bind peptides is a unique feature of grp170.

Glucose-regulated proteins (grps)[1] are molecular chaperones that reside in the endoplasmic reticulum (ER). Grp expression is significantly increased by several stress conditions such as anoxia, reductive stress, calcium ionophores, and glucose starvation. Heat shock does not significantly increase grp levels. The most prominent grps are designated by their molecular mass in kilodaltons and contain grp78 (also called the B-cell immunoglobulin binding protein or "BiP"), grp94 (also called gp96), and grp170. Of this group, grp170 has been the least well studied, and little is known about its chaperoning properties. Grp170 is predicted to share homology in amino acid composition with members of the hsp70 and hsp110 stress protein families. However, a phylogenetic analysis predicts that grp170 is in a family distinctly different from hsp110 and hsp70 (*1*−*3*). Grp170 family members have been found in all eukaryotic cells thus far examined, including *Saccharomyces pombe, Saccharomyces cerevisiae*, and mammalian cells (*1*−*4*). Initial studies with mammalian B-cell hybridomas demonstrated that grp170 associates with immunoglobulin chains as well as with grp78 and grp94, suggesting that it may have a role in the assembly/folding of secretory proteins (*5*). It was also shown that grp170 is involved in signal recognition particle-independent posttranslational translocation of proteins into the ER in yeast and mammals (*6*−*8*). In addition, it was demonstrated that grp170 helps to transport peptides into the ER via the transporter associated with antigen processing (TAP) (*9*), suggesting that grp170 is involved in peptide trafficking in the antigen presentation pathway.

Despite these studies the molecular chaperoning functions of grp170 have not been described in detail. Many studies of hsp70 family members have appeared and are summarized elsewhere (*10*). In our previous studies we have focused on hsp110, a representative of the second family of hsp70-related proteins (*11*). We have shown that hsp110 is highly efficient in binding heat-denatured substrate proteins (e.g., luciferase) and prevents aggregation. In the presence of rabbit reticulocyte lysate (RRL) and ATP, hsp110 refolds denatured protein to a greater degree than hsc70 (*11*). Domain deletion mutant studies with hsp110 have shown that binding does not occur at the N-terminal, putative ATP binding domain (*12*). The predicted β-sheet domain of hsp110 was found to be necessary for binding and stabilizing luciferase under heat shock conditions (*12*).

Here we characterize the chaperoning properties of mouse grp170. We demonstrate that grp170 binds heat-denatured luciferase, prevents its aggregation, and efficiently holds luciferase in a folding-competent state. Domain deletion mutants of grp170 were created to determine the contribu-

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[1] Abbreviations: grp, glucose-regulated protein; hsp, heat shock protein; grp170, 170 kDa glucose-regulated protein; gp96, 96 kDa glucose-regulated protein; hsp110, 110 kDa heat shock protein; hsp70, 70 kDa heat shock protein; A, ATPase domain of grp170; AB, ATPase and β-sheet domains of grp170; ABL, ATPase, β-sheet, and long acidic loop domains of grp170; BLH, β-sheet, long acidic loop, and α-helical domains of grp170; LH, long acidic loop and α-helical domains of grp170; H, α-helical domain of grp170; RRL, rabbit reticulocyte lysate; ova, ovalbumin; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Endo H, endoglycosidase H.

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tions of predicted structural domains. As in hsp110 the N-terminal ATP binding domain of grp170 is dispensable for its in vitro chaperoning functions. However, unlike hsp110 or hsp70, grp170 is found to have two clearly defined peptide binding domains. The first domain is similar to the β-sheet region of hsp70 and hsp110, and the second domain is similar to the C-terminal α-helical domains of hsp70 and hsp110. It is the chaperoning ability of this second domain that differs from hsp70 and hsp110. These data suggest that grp170 may be able to bind two proteins at once and also support its previously proposed role (*13*) in chaperoning and assembly of multiproteins such as immunoglobulin complexes in the ER.

EXPERIMENTAL PROCEDURES

Cloning and Expression of Mouse Grp170. Grp170 cDNA (mouse) was cloned into a baculovirus transfer vector containing the promoter for transcription and sequences for homologous recombination and selection. A polyhistidine tag sequence was added internally prior to the stop codon, after the KNDEL sequence, since an N-terminal polyhistidine sequence in the vector is removed after the protein is translocated into the ER. The transfer vector was cotransfected with replication-deficient baculoviral DNA into Sf21 insect cells (Clontech BacPAK baculovirus expression system, Palo Alto, CA). The recombinant virus selection and amplification of virus steps were performed according to the manufacturer's instructions. After amplification of virus several times, the virus was infected into suspension cultures of Sf21 cells (MOI = 5−10). After a 3−4 day infection (depending upon individual mutants), cells were pelleted and stored at −70 °C until purification. Cells were lysed in 20 mM Tris-HCl (pH 7.9), 0.5 M NaCl, 5 mM imidazole, 0.1% Nonidet P-40, and protease inhibitor cocktail (Promega, Madison, WI) and incubated for 30 min on ice. The lysate was centrifuged at 10000*g* for 1 h, and the supernatant was centrifuged at 100000*g* for 2 h. His-grp170 was purified from the supernatant on Ni^{2+}-nitrilotriacetic acid−agarose columns (QIAGEN, Inc., Valentica, CA) following the manufacturer's instructions. Briefly, the supernatant was loaded on the column and washed with buffer containing 20 mM Tris-HCl, 0.5 M NaCl, and 20 mM imidazole, pH 7.9. The imidazole concentration was increased to 50 mM by 10 mM steps. Grp170 was eluted with wash buffer containing 300 mM imidazole. The eluted proteins were dialyzed against phosphate-buffered saline, pH 7.9, for 16 h. Proteins were concentrated with Centriplus (Millipore, Bedford, MA) or Vivaspin20 (Vivascience, Westford, MA) ultrafiltration columns with a molecular mass cutoff of 10 kDa. Proteins were quantified using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard.

Plasmid Constructions for Grp170 Mutants. All plasmid constructs were made to produce C-terminal 6-histidine-tagged proteins. All grp170 mutants have a 34 amino acid leader sequence and 6-histidine sequence before the stop codon. For the polymerase chain reaction (PCR), the following primers were synthesized. Each primer sequence contained proper restriction enzyme recognition sequences, which are underlined and whose names are indicated in parentheses. Primers I, II, III, and IV are 5′ (or forward primers for PCR) and IV, V, VI, and VII are 3′ (or backward primers for PCR). Bac1 and Bac2 primers are sequences from the pBacPAK vector each 5′ and 3′ of multiple cloning sites. For the constructions of BLH (430−999 AA), LH (600−999 AA), and H (715−999 AA) mutants, PCR was performed with primer pairs of primer I and primer Bac2, primer II and primer Bac2, and primer III and primer Bac2, respectively. The PCR products were digested with appropriate restriction enzymes, and the fragment was ligated into the BacPAK His-I vector (Clontech), which was also digested with the same restriction enzymes used for the digestion of the PCR product. The leader sequence was synthesized by PCR with primer pairs of primer Bac1 and promer IV, digested with *Sal*I and *Bam*HI restriction enzymes, and ligated into the same vector. For the construction of A (1−430 AA), AB (1−600 AA), and ABL (1−715 AA) mutants, PCR was performed with primer Bac1 and primer V, primer Bac1 and primer VI, and primer Bac1 and primer VII. All three PCR products were amplified and extended by a first round of PCR with primer Bac1 and primer VIII and extended again to the 3′ direction by another PCR with primer Bac1 and primer IX to code KNDEL, the ER resident sequence, and the polyhistidine sequence. The PCR products were digested with *Bam*HI and *Xba*I restriction enzymes, and the digested fragments were ligated into the pBacPAK His-1 vector, which was also digested with the same restriction enzymes. Following are the sequences of each primer: Bac1, 5′-ACCATCTCGCAAATAAATAAG-3′; Bac2, 5′-ACAACTCACAGAATCTAGCG-3′; primer I (*Sal*I), 5′-AGCAAAGTCGACAAGGTGAAGCCGTTTGTT-3′; primer II (*Sal*I), 5′-AATGGTGTCGACGCTGCACAG-GAGGAAGA-3′; primer III (*Sal*I), 5′-TCCTGAGGTCGA-CAAAAAGCCGAAACCTGC-3′; primer IV (*Sal* I), 5′-GCCCAGGTCGACAGACATCACAGCCAACG-3′; primer V, 5′-TAGTTCATCGTTCTTGAAGGCCTTGCTGAGCG-CCG-3′ (KNDEL); primer VI, 5′-TAGTTCATCGTTCT-TATCAGTACCATTCTCTGG-3′ (KNDEL); primer VII, 5′-TAGTTCATCGTTCTTATCCTCTGGCAAGTCAGGCA-3′ (KNDEL); primer VIII, 5′-GGTGATGATGATGAGAT-CCTCTTAGTTCATCGTTCTT-3′ (KNDEL); primer IX (*Xba*I), 5′-CCAGATCTTCTAGATTAGTGATGGTGAT-GATGATGAG-3′.

The expression and purification of grp170 mutants were performed in the same manner as full-length grp170.

Prediction of Grp170 Secondary Structure and Definition of Domains. Prediction of the secondary structures for grp170 and structural domains was performed using the consensus of results from the eight secondary structure prediction programs implemented on the Network Protein Sequence Analysis (NPS@) server (*13*), using default parameters. The predicted structure for the intact grp170 molecule was structurally aligned with the predicted structure of hsp110 and the crystallographically determined structure for DnaK (*3*, *12*). The structural domains of the proteins were delineated by comparison with those defined for hsp110 (*12*).

Plasmid Constructions for Hsp110 and Hsp70 Mutants. Plasmids for hsp110 LH and H mutants were constructed on the basis of the model of Oh et al. (*12*) with a C-terminal 6-His tag. For the construction of hsp110 LH and H mutants, PCR was performed with primer pairs of primer X and primer XII and of primer XI and XII, respectively. Primers X and XI are forward primers for PCR and primer XII is a backward primer: primer X (*Bam*HI), 5′-GACGACGG-GATCCTCTGTCGAGGCAGACATGGA-3′; primer XI

(*Bam*HI), 5′-AGTTAGGGATCCAGAGACCTTCTTAACATGTAC-3′; primer XII (*Hin*dIII), 5′-CAGCGCAAGCTTACTAGTCCAGGTCCATATTGA-3′. The PCR products were digested with appropriate restriction enzymes and ligated into the pET21a(+) vector (Novagen), which was digested with the same restriction enzyme and has a C-terminal polyhistidine sequence.

The hsp70 H mutant was constructed by PCR with the appropriate primer set: primer XIII (*Bam*HI), 5′-ACCAACGACAGGATCCGCCTGAGCAA-3′; primer XIV (*Eco*RI), 5′ATCTGCAGAATTCCTACTAATCCAC-3′. The PCR product was digested with *Bam*HI and *Eco*RI restriction enzymes, and the fragment was ligated into the pRSETC vector (Invitrogen, Carlsbad, CA). The constructs were transformed into *Esherichia coli* BL21(DE3) cells. The transformed *E. coli* were cultured at 37 °C until absorbance at 600 nm reached 0.6. Isopropyl 1-thio-β-D-galactopyranoside (IPTG) (1 mM) was added, and cells were further cultured for 5 h at 30 °C. The purification method is the same as used for grp170 purification.

Circular Dichroism Spectra. Grp170 and the mutants were prepared as described above. Equimolar samples (1.18×10^{-7} M) of grp170 and the domain deletion mutants ABL, H, LH, BLH, and A were prepared in sterile PBS, pH 7.5, and kept at 4 °C until needed approximately 2 h later. Each sample was loaded into a clean quartz cuvette and placed in the sample holder of a Jasco J-715, circular dichroism spectrophotometer (Jasco, Easton, MD). The sample compartment was heated with a Peltier temperature control unit. The samples were allowed to equilibrate to 37 °C for approximately 1 min before a run was initiated. The samples were probed with 250−200 nm light. The final spectrum is an average of four individual scans.

Endoglycosidase H (Endo H) Digestion. Recombinant proteins were incubated in 50 mM sodium citrate, pH 5.5, with or without 5 milliunits of Endo H (Roche) at 37 °C for 3 h. Samples were then resolved in 10% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and stained with GelCode Blue reagent (Pierce Biotechnologies).

Luciferase Aggregation Assay. Luciferase (150 nM) (Sigma, St. Louis, MO) and various test proteins at the same molar ratio were incubated in buffer containing 25 mM HEPES (pH 7.9), 5 mM magnesium acetate, 50 mM KCl, and 5 mM β-mercaptoethanol at 43 °C for 30 min. Protein aggregation was determined by an increase of the optical density at 320 nm. As negative controls, ovalbumin (ova) and bovine serum albumin were used. At the end of the heating period, the solution was centrifuged at 16000*g* for 15 min, and soluble and pellet fractions were separated. Electrophoresis, transfer, and immunoblotting were performed as described previously by Lin et al. (*14*). Briefly, The soluble and insoluble proteins were analyzed using 10%, reducing SDS−PAGE (pH 8.8). The proteins were transferred to nitrocellulose. Mouse anti-luciferase antibody (Promega, Madison, WI) was used as the primary antibody in immunoblotting.

Detection of the Interaction between Luciferase and Grp170 by Immunoprecipitation. Luciferase (150 nM) was incubated with grp170 (150 nM) in buffer used for aggregation experiments at 4 or 43 °C for 30 min and then chilled on ice. Anti-grp170 antibody was added to the luciferase solution, and the buffer was adjusted to radioimmune precipitation buffer (RIPA; phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) and incubated for 1 h at 4 °C followed by incubation with protein A−Sepharose (Promega) for 16 h at 4 °C. The protein A−Sepharose pellet was collected and washed six times. The samples were analyzed with 10% SDS−PAGE and probed by Western analysis as described above.

Luciferase Refolding Assay. Luciferase was heated in refolding buffer (25 mM HEPES, pH 7.6, 5 mM $MgCl_2$, 2 mM dithiothreitol, and 2 mM ATP) at 43 °C for 30 min. Heated luciferase was diluted 100-fold into 60% RRL (Promega) refolding buffer and incubated at 30 °C for 2 h. For the measurement of activity the luciferase solution was further diluted 5-fold into 25 mM HEPES, pH 7.6, containing 1 mg/mL bovine serum albumin. Then 10 μL of this was added to 100 μL of luciferase assay solution (Promega). Luciferase activity was measured with a Lumat LB9501 luminometer (Berthold Technologies).

RESULTS

Expression of Mouse Grp170. For these studies we chose to use mouse grp170. Since only partial sequence information was available for mouse, we cloned the full-length mouse cDNA for grp170 using primers designed from the hamster sequence. The sequence of the grp170 cDNA from hamster was determined previously (*1*). The full-length mouse sequence was submitted to GenBank (accession number AF228709). The full-length cDNA encodes a protein of 999 amino acids which is 95.5% identical to the hamster sequence, the features of which are described elsewhere (*1*).

Recombinant grp170 protein was prepared using the baculovirus-based eukaryotic expression system. To recover the protein, we introduced a C-terminal His-tag sequence, necessitated by the fact that the N-terminal signal sequence is cleaved after translocation into the ER. This His tag did not interfere with the function of the KNDEL. Grp170 isolated from mammalian cells or tissues migrates as a single band of approximately 170 kDa on SDS−PAGE. When digested by endoglycosidase H, grp170 is observed to migrate in SDS−PAGE at approximately 150 kDa (*14*). Grp170 purified from the Sf21 insect cells used here yields two bands on SDS−PAGE, one of each of these sizes (this is evident in Figures 1C and 4). The higher molecular mass band comigrates on SDS−PAGE with glycosylated grp170 and the lower with the nonglycosylated form (expressed in *E. coli*; data not shown). These data suggest that Sf21 cells are unable to glycosylate all grp170 in this high output expression system.

Grp170 Inhibits Heat-Induced Protein Aggregation by Selectively Binding Denatured Protein. Using the recombinant grp170, we examined its chaperoning functions using in vitro assays. Luciferase was used as a sample substrate to examine the ability of grp170 to bind to and inhibit heat-induced aggregation. It was observed that grp170 is highly efficient in inhibiting the heat-induced aggregation of luciferase at 43 °C, preventing aggregation at a 1:1 substrate to chaperone molar ratio (Figure 1A). This is similar to results from our previous studies with hsp110 (*11*). These data indicate that both of these large chaperones are highly efficient holders of denatured protein, significantly more so than members of the hsp70 family (Figure 1A and data not

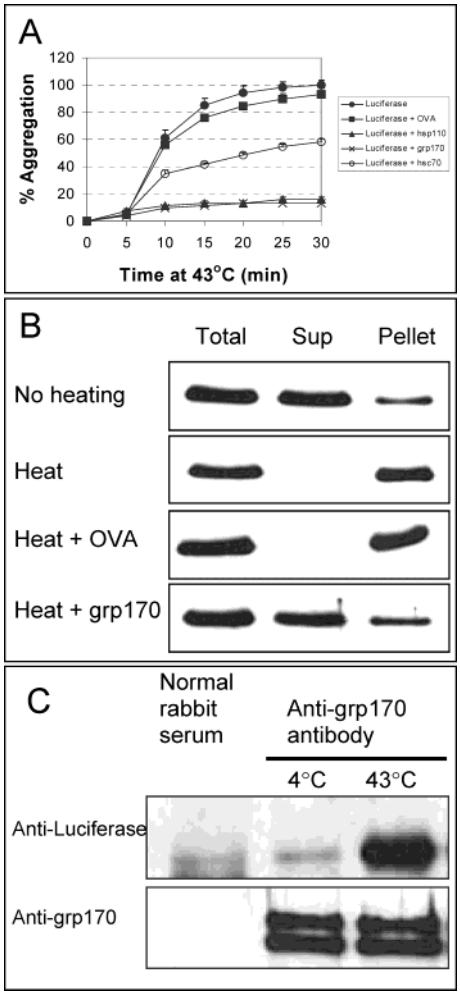

FIGURE 1: Effect of grp170 on the inhibition of protein aggregation in vitro. Panel A: Grp170, hsp110, and hsc70 inhibit heat-induced luciferase aggregation as determined by light scattering. Hsp110 and grp170 function more efficiently than does hsc70 as protein stabilizers. Ova was used as a control protein. Panel B: Western blot analysis of soluble luciferase in the supernatant (sup) and aggregated luciferase in the pellet (pellet) after centrifugation at 16000*g*. Panel C: Coimmunoprecipitation analysis using an antibody against grp170, followed by Western blotting and probing with antibody against luciferase. This demonstrates that grp170 coprecipitates (primarily) heated luciferase but not luciferase incubated at 4 °C. Grp170 in the immune complex is also shown (probed with anti-grp170 antibody).

shown). As controls, bovine serum albumin and ova (both aggregation resistant to nearly 80 °C) do not prevent luciferase aggregation. Grp170 itself denatures and aggregates at approximately 70 °C. Additionally, 1 mM ATP did not increase the luciferase binding activity of grp170 (data not shown). These results were confirmed by centrifugation and analysis of the amount of luciferase present in the supernatant and pellet (Figure 1B).

To determine if the observed inhibition of luciferase aggregation results from binding of grp170 to luciferase, an anti-grp170 serum was used to precipitate the putative grp170−luciferase complex. A Western blot analysis of the immunoprecipitate showed that the anti-grp170 antibody also precipitates heat-denatured luciferase (Figure 1C). These data indicate that grp170 is a highly efficient molecular chaperone that binds to and prevents aggregation of denatured luciferase, presumably by binding to and shielding exposed hydrophobic

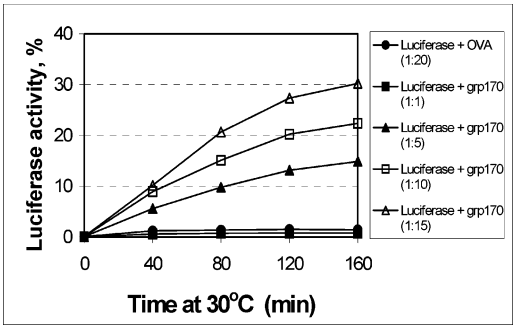

FIGURE 2: Luciferase bound to grp170 can be refolded by RRL. Grp170 maintains luciferase in a folding-competent state during heating, and when they are incubated with RRL, luciferase regained some activity. Molar ratios of luciferase to grp170 are indicated. While grp170 maintains luciferase in a folding-competent state after heat shock, it cannot refold this protein by itself.

residues. This chaperoning property of grp170 is not restricted to luciferase and can be reproduced using other protein substrates, specifically citrate synthase and the melanoma antigen gp100 (data not shown).

Grp170 Maintains Denatured Protein in a Folding-Competent State. Although grp170 prevents aggregation of heat-shocked luciferase and maintains it in a soluble state, luciferase irreversibly loses its enzymatic activity following heating. Addition of 2 mM ATP to the holding/folding buffer (containing heat-denatured luciferase and grp170) and a 3 h incubation at 30 °C did not restore luciferase activity. RRL has previously been reported to be an optimal refolding buffer, possibly due to the presence of necessary cochaperones. Thus we examined whether grp170 could refold luciferase into an enzymatically active form by addition of RRL (*15*−*17*). RRL itself, in the absence of exogenous chaperones, does not reactivate heat-denatured luciferase, but grp170 in the presence of 60% RRL at 30 °C can restore about 30% activity after 3 h (Figure 2). Restoration of luciferase activity also requires ATP. These data indicate that grp170 alone cannot restore protein function but can hold the protein and maintain it in a folding-competent state. An increased molar ratio of grp170 to luciferase during heating is required for optimal refolding as compared to inhibition of aggregation as described above (Figure 2).

Previous studies on hsc70 have shown that a limited degree of refolding of denatured protein can be obtained by coincubation of the target protein with hsc70 and Hdj-1, a mammalian DnaJ homologue (*18*). Hdj-1 is a cytosolic J-protein and therefore would not interact under normal physiological conditions with grp170. However, J domains are conserved between cytosolic and ER proteins (*19*, *20*). No active luciferase was obtained with coincubation of grp170 and Hdj-1 (data not shown). However, other unidentified, specialized ER J-proteins may be necessary for grp170 to refold denatured proteins.

Predicted Structure of Grp170 and Purification of the Targeted Mutants. We wished to determine the structural domains of grp170 involved in chaperoning activity. Since a crystallographic structure for grp170 is unavailable, we approached the design of deletion mutants of grp170 using the same homology modeling protocol previously used to determine the functional domains of hsp110 (*12*). We used the consensus predictions of eight programs as implemented by Combet et al. (*13*) to predict the secondary structure of

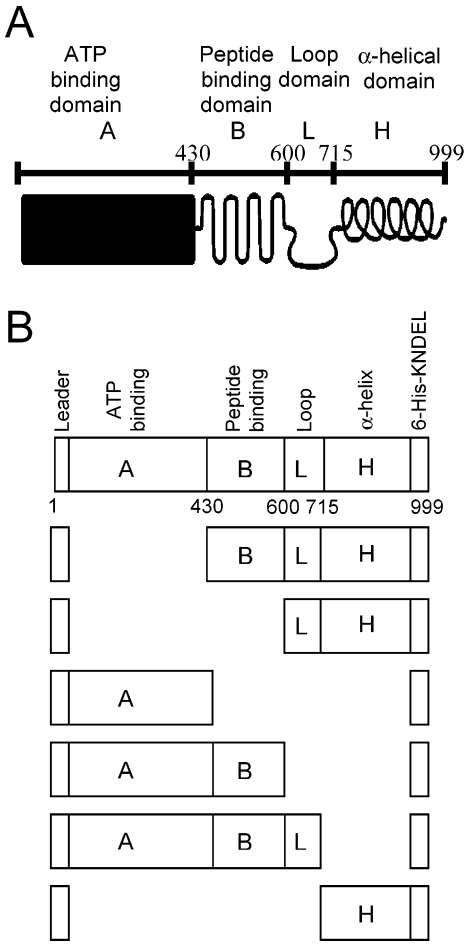

FIGURE 3: Construction of grp170 deletion mutants. Panel A: Schematic illustration of the predicted domains of grp170. The domains examined are as follows: A, ATP binding domain; B, β-sheet (putative peptide binding) domain; L, acidic loop domain; H, α-helical domain. Panel B: The grp170 deletion mutants constructed for this work are indicated. The N-terminal leader sequence for ER localization, C-terminal 6-His tag for purification, and KNDEL sequence for ER retention are also indicated.

grp170. We also used structural and amino acid alignments with DnaK and hsp110 to determine an approximate secondary structure map for grp170 (*3*). The putative structure for grp170 (Figure 3A) consists of an N-terminal, 40 kDa globular, ATP binding domain (referred to as A) which has 30% identity with the ATP binding domain of DnaK. This is followed by a β-sheet domain (B) consisting of approximately seven β-strands, followed by a long acidic loop domain (L) which is unique to grp170. The C-terminal domain (H) is somewhat similar to the α-helical lid in DnaK/hsp70. The H domain is predicted to form approximately five α-helices joined by random coils and is followed by a long C-terminal loop which ends in KNDEL.

We designed several deletion mutants on the basis of these features (Figure 3B): (1) an ATP binding domain deletion (BLH) mutant (residues 430−999), (2) an ATP binding domain and β-sheet domain deletion (LH) mutant (residues 600−999), (3) an ATP binding domain only (A) mutant (residues 1−430), (4) an ATP binding domain and β-sheet domain (AB) mutant (residues 1−600), (5) a helical domain deletion (ABL) mutant (residues 1−715), and (6) a helical domain only (H) mutant (residues 715−999). These mutants were constructed from the cloned mouse grp170 cDNA using

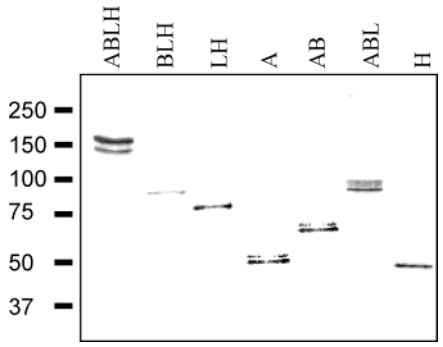

FIGURE 4: Characterization of purified grp170 and mutant proteins. Grp170 and its deletion mutants were expressed using the baculovirus expression system and purified using Ni^{2+}-nitrilotriacetic acid−agarose columns. Recombinant proteins were resolved and analyzed using 10% reducing SDS−PAGE.

specific primers that were used to set the boundaries of each mutant by introducing engineered restriction sites. The gene segments were amplified by PCR, digested with the appropriate restriction enzymes, and ligated into vector DNA. The coding sequences were introduced into a baculovirus vector for expression in insect Sf21 cells as described in Experimental Procedures. Mutant proteins were extracted from infected cells and tested for purity and size by SDS−PAGE (Figure 4). The A, AB, and H mutants were sparingly soluble. Only the soluble fractions were used for the chaperoning activity assay. When purified and analyzed by 10% SDS−PAGE (pH 8.8), the apparent molecular mass determined for each mutant was often different than that expected from the amino acid composition. The mutants containing the loop domain (including the full-length protein) are larger than predicted by more than 20 kDa. Sequence analysis shows that the loop domain is rich in acidic amino acid residues; however it is unclear as to whether this property affects gel migration. Moreover, mutants A, AB, and ABL, as well as the full-length protein (ABLH), produced two bands in SDS−PAGE gels while BLH, LH, and H mutants yielded only one band (Figure 4). Since grp170 is glycosylated (*14*), this implies that the A domain is glycosylated. There are two glycosylation sequences in the A domain (155−158 NYSR and 222−225 NSTA) of nine predicted for the entire molecule. To verify that it is only the A domain that is glycosylated, full-length grp170 (ABLH) and the BLH mutant were digested with Endo H (Figure 5). The protein doublet present for the full-length grp170 resolved to a single band after digestion with Endo H. However, the migration of the protein expressed by the BLH mutant remained unchanged, demonstrating that high mannose oligosaccharides are localized in the ATP binding (A) domain. This explains why grp170 and mutants containing the A domain exhibit two bands.

The structures of the ABL, BLH, LH, H, and A domain deletion mutants were examined using circular dichroism (CD). Circular dichroism spectra are interpreted as linear combinations of CD spectra from individual peptides; thus we attempted to reconstruct the spectrum of full-length grp170 from the mutants (*21*). When the CD spectra of the ABL and H domains are added together, the CD spectrum of full-length grp170 is accurately reconstructed (Figure 6). Furthermore, the spectrum of the α-helical H domain is highly consistent with CD spectra for α-helical peptides,

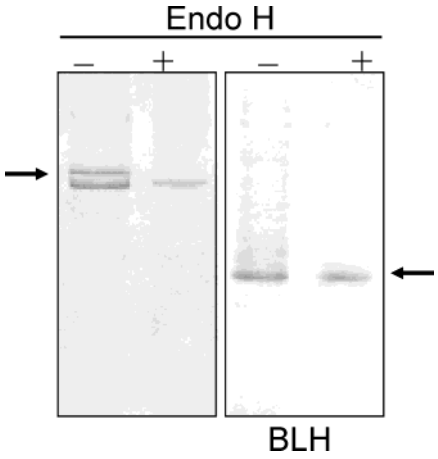

FIGURE 5: Endoglycosidase H digestion of wild-type grp170 (ABLH) and the BLH mutant. Full-length grp170 and BLH mutant were treated (+) or not treated (−) with Endo H as indicated. All samples were resolved by SDS−PAGE and visualized by GelCode Blue staining.

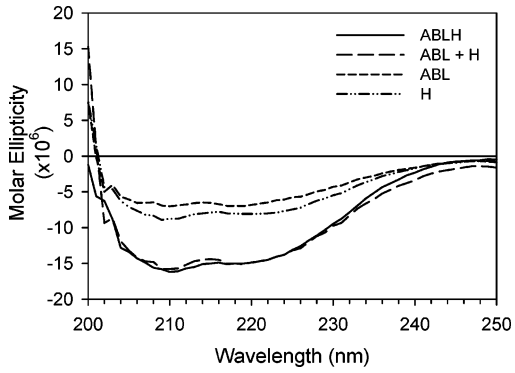

FIGURE 6: Circular dichroism (CD) spectra of full-length grp170 and its domain deletion mutants. Equimolar grp170 (solid line) and the domain deletion mutants, ABL (medium dash line) and H (dash dot line), were heated and equilibrated to 37 °C followed by analysis with a CD spectrophotometer at 250−200 nm. It was seen that adding the ABL and H spectra regenerates the full-length grp170 spectrum (large dash line).

Table 1: Comparison of α-Helix, *β*-Sheet, and Coil Content of Full-Length Grp170 and Domain Deletion Mutants, As Determined by Analysis of CD Spectra, with Values Predicted from the Primary Structure[a]

	α-helix (%)		*β*-sheet (%)		coil (%)	
	CD	2D	CD	2D	CD	2D
ABLH	37	39	13	10.10	49	46
ABL	30	35	13	12.70	55	46
H	31	53	13	0.35	56	42

[a] 2D values do not add up to 100% due to residues in ambiguous states.

having two easily identifiable negative peaks at 220 and 208 nm (*21*). We have used the program K2D (*22*) to analyze the CD spectra of the full-length grp170 (ABLH) and the H and the ABL mutants. The relative content of α-helix, *β*-sheet, and random coil in the full-length protein and the mutants, determined from this analysis, agrees closely with the relative content of these secondary structures predicted from the protein's primary structure (Table 1). The only significant deviation is in domain H, where CD detects less helix, more *β*-sheet, and less coil than the predictions. These data demonstrate that the ABL and H mutants closely retain the structure that they possess within full-length grp170. Since the H mutant appears to be folded correctly, we subtracted its CD spectrum from that of the LH mutant and achieved a spectrum that is consistent with randomly coiled peptide chains (single positive peak starting at about 217 nm). The L domain of grp170 has previously been predicted to consist of 85% random coils. This suggests that the LH mutant structure is also nearly identical to the LH domains within the native grp170 structure. The A and AB mutants did not give interpretable CD spectra, probably because both proteins aggregate under the conditions used for analysis.

Identification of Domains Required for the Chaperoning Activity of Grp170. We next examined the chaperoning activities of the grp170 mutant proteins just outlined. The data from these experiments are shown in Figure 7. We observed that neither the ATP binding domain (A) nor the ATP binding domain plus the *β*-sheet domain (AB) mutants prevented the heat-induced aggregation of luciferase (Figure 7B,C). However, the mutant lacking the ATP binding domain (BLH) was observed to exhibit the full ability to bind to and stabilize heat-denatured luciferase (Figure 7E). The additional deletion of the *β*-sheet domain (i.e., mutant LH) can still function in this capacity as a chaperone (Figure 7F). This mutant lacks the *β*-sheet domain that is critical to the chaperoning function of hsp110 (*11*), hsp70 (*23*, *24*), and DnaK (*25*). In addition, mutant ABL, lacking the H domain, was also found to exhibit holding activity (Figure 7D). This demonstrates that both ABL and LH exhibit a strong ability to bind to luciferase and inhibit its heat-induced aggregation. Last, we made an H, α-helical domain only mutant. The H domain alone also prevented luciferase aggregation as effectively as did wild-type grp170 (ABLH), BLH, and LH (Figure 7G). That LH and H mutants possess equivalent activity suggests that H is the active element of the LH mutant. Moreover, the ABL mutant is active while AB is not, suggesting that the L region is necessary for the *β*-sheet (B) region to exhibit activity. This is similar to our earlier studies with hsp110 when some C-terminal sequence to the B domain was required for function (*12*). It is possible that L (which may possess some structure in grp170) is acting as the C-terminal lid described by Zhu et al. (*25*). These studies demonstrate that grp170 has two domains that can bind to and prevent the heat-induced aggregation of luciferase and suggest that one is the BL region while the second is the H domain. This observation is not restricted to luciferase as the substrate protein. These chaperoning activities determined by light scattering were confirmed by centrifugation experiments (Figure 8A). Mutants BLH, LH, ABL, and H maintained luciferase in solution while A and AB mutants did not.

We next repeated the luciferase refolding assay, as described above, with the mutants (Figure 8B). When we incubated luciferase with LH and H domains (without the *β*-sheet domain) and added RRL, heat-inactivated luciferase not only recovered activity but also did so to a significantly greater degree than was obtained with wild-type grp170, i.e., ABLH (for LH vs ABLH, $p = 0.0017$; for H vs ABLH, $p = 0.00021$). Recovery of luciferase activity with other mutants reflected their holding ability. Therefore, LH and H mutants not only hold luciferase in a folding competent state but also

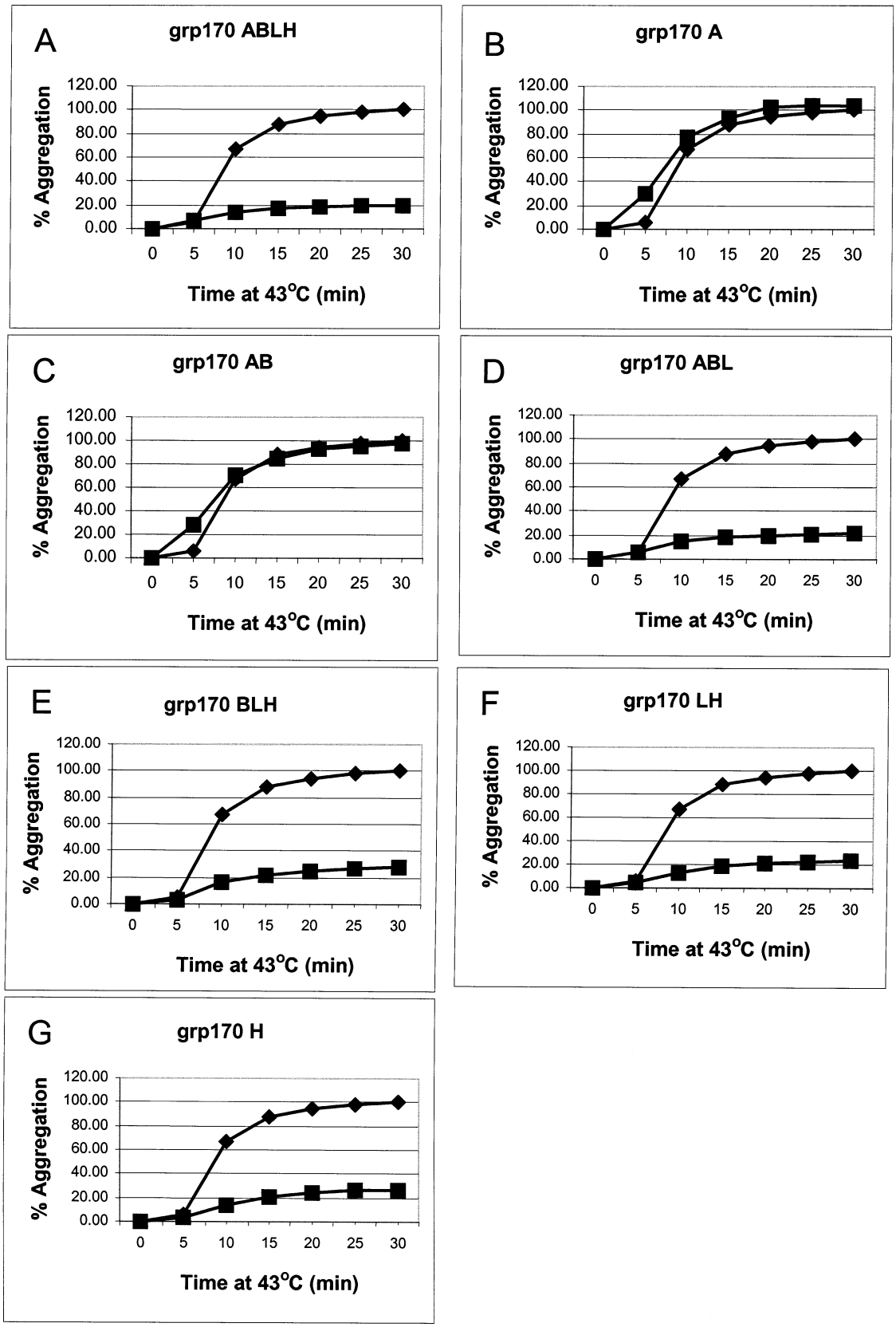

FIGURE 7: Analysis of the protein stabilization efficiency of full-length grp170 (ABLH) (panel A) and the grp170 domain deletion mutants (panels B−G). Luciferase aggregation is shown in each panel (diamonds) for comparison.

do so much more effectively than does full-length grp170.

Last, to further analyze the protein chaperoning and refolding properties of the grp170 H domain, we prepared analogous H domains from hsp110 and hsp70. The ability of full-length grp170 and all three H domains to stabilize luciferase is shown in Figure 9. It is seen that only the grp170 H domain alone is able to protect luciferase from heat-induced aggregation.

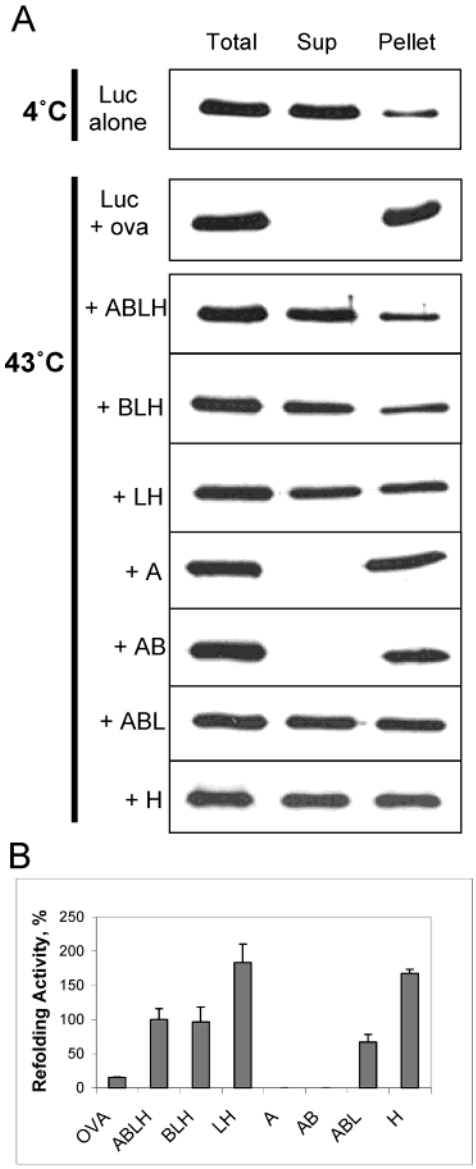

FIGURE 8: Panel A: Individual panels show the aggregated and soluble fractions of luciferase after heating alone or in the presence of full-length grp170 (ABLH) or the domain deletion mutants. Panel B: Ability of grp170 (ABLH) or the domain deletion mutants to refold luciferase into an active form. LH and H mutants are significantly more efficient than is the full-length ABLH protein. All experiments were performed at a 10:1 mole ratio of grp170 (or mutant):luciferase and in the presence of RRL. Student's unpaired *t*-test was used for statistical analysis. Data are presented as the mean ± standard deviation from at least three experiments (for LH vs ABLH, $p = 0.0017$; H vs ABLH, $p = 0.00021$).

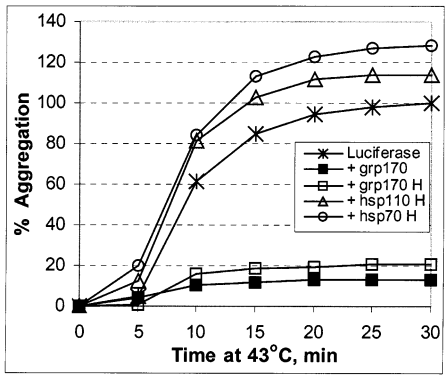

FIGURE 9: Comparison of the chaperoning activity of the H domains of hsp70, hsp110, and grp170. H domains of all three proteins were examined using the luciferase aggregation assay in vitro. The H domains of hsp70 (open circles) and hsp110 (open triangles) were unable to prevent luciferase aggregation while the H domain of grp170 (open squares) and full-length grp170 (filled squares) could stabilize luciferase with comparable efficiency.

DISCUSSION

There are two major groups of stress proteins: (1) the hsps, which are induced by heat, ethanol, oxidative stress such as occurs during reperfusion injury, among numerous other environments and conditions; and (2) the grps, which are induced by conditions including glucose deprivation, chronic hypoxia and reductive stress, inhibition of glycosylation, and interference with calcium homeostasis (*1*, *3*). Grps reside in the ER while the hsps reside in the cytosol, nucleus, and mitochondria, reflecting the differential stress sensitivities of different cellular compartments. Grp78 (BiP) is a well-studied member of this group of molecular chaperones and is highly homologous to hsp70 (*26*). Grp94 (gp96) is homologous to cytosolic hsp90 (*27*). Grp170 is homologous with hsp110 as well as with hsp70 and grp78. Grp170 proteins are found in all organisms examined from yeast to man and upon sequence analysis group into a single family (*1*−*3*). This family is related to, but divergent from, the hsp70 family which itself is a distinct sequence group or family. Hsp110s from numerous organisms also define a unique family which has been called the hsp110/SSE family (*28*). Thus, these three stress protein families, all interrelated but distinctly different from one another, make up what has been termed the hsp70 superfamily (*3*). Interestingly, grp78 is observed to group with the hsp70s and does not represent a unique family, while grp170 does (*1*, *3*, *6*).

The chaperone functions of hsp70 have been intensively studied (*24*, *29*−*32*), and the chaperoning properties of hsp110 have also been described (*11*, *12*). Grp170 has been shown to associate with and presumably chaperone immunoglobulin light chains and thyroglobulin in vivo (*14*, *33*). It also is associated with peptide transport into the ER (*8*, *33*). Its ability to bind to and chaperone protein substrates has not been examined in vitro. In this paper we show that recombinant grp170 is capable of preventing the aggregation of heat-denatured luciferase and maintains it in a folding-competent state in vitro. Grp170's behavior in this respect is similar to that of hsp110. However, grp170 binds ATP readily, while hsp110 has a very weak ATP binding ability (refs *11* and *12* and unpublished data). Despite this major difference, neither hsp110 nor grp170 require ATP for binding unfolded protein, and both chaperones require RRL to promote refolding of the bound protein, indicating the necessity of a cochaperone like Hdj-1. We note that RRL contains cytosolic components while grp170 is an ER chaperone. Nonetheless, RRL is capable of refolding heat-denatured luciferase held in a complex with grp170. That J domains are conserved between cytosolic and ER J proteins suggests that some similarities in folding pathways exist in each organelle.

Both proteins contain the predicted β-sheet domains which are homologous to the peptide binding domain of DnaK (*24*). However, the ABL mutant of hsp110 is inactive in binding luciferase, while the comparable ABL form of grp170 has chaperoning activity. This may be related to the larger L domain of grp170. Importantly, the C-terminal α-helical

region of grp170 (H) also binds denatured polypeptides and holds them in a folding-competent state. This secondary binding site is not present in hsp70 and hsp110 or, if present, requires additional factors for activity.

In the structure of DnaK, the β-sandwich region (B) serves as the peptide binding site, whereas the helix−turn−helix structure (H), poised above the β-sandwich, is thought to regulate entry and/or exit of the peptide substrates (*25*). The structure of hsp110 has been previously modeled, using the structure of DnaK, and the data obtained indicate that the predicted β-sheet domain of hsp110 is again an essential element responsible for its peptide binding properties (*11*). However, hsp110 is much larger than DnaK and hsp70, due to the expansion of the C-terminal half of the protein (L and H domains). The conserved but expanded version of the lid in the hsp110 family may represent an elaboration on the functional properties of the homologous DnaK structure, and hsp110 has been shown to be significantly more efficient than hsp70 in stabilizing heat-denatured protein. The results reported here for the grp170 mutants share some similarities with our earlier studies with hsp110. Once again, grp170 is notably more effective than hsc70 in stabilizing heat-denatured protein. However, unlike hsp110, the H domain of grp170 can also act as a potent substrate binding domain. Grp170 (residues 700−900) shares 19% identity with the C-terminal 200 amino acids of bovine hsc70 and 18% identity with the corresponding segment of grp78 but exhibits 40% identity with hsp110 (residues 600−800) (*3*). However, the grp170 H domain has a hydrophobic region spanning residues 820−900 while the (predicted) structurally equivalent region of hsp110 appears to be somewhat more hydrophilic.

Although much more information will be required to understand the structural properties of grp170's C-terminal domain which are responsible for its ability to bind denatured protein and hold it in a folding-competent configuration, we suspect that a key may lie in the possibility of the C-terminal helices (which contain heptad repeats) forming a coiled coil. The coiled-coil structure is commonly involved in protein−protein binding interactions. For example, prefoldin (PFD) is a eukaryotic chaperone protein and consists of an $\alpha_2\beta_4$ hexamer. The α and β chains are not identical but have similar structure and form a coiled-coil structure. In PFD the crevice between helixes in the coiled coil has a hydrophobic property and forms the hydrophobic unfolded protein binding region (*35*−*37*). So it is plausible that the grp170 H domain can provide a peptide binding region in a coiled-coil structure. Furthermore, X-ray and NMR structures of DnaK indicate that it forms dimers with the C-terminal helices from the two molecules combining to form a coiled-coil structure (*25*). However, the question still remains as to why grp170 in the ER has two protein binding domains while cytosolic hsp110 does not. It is also possible that, in vivo, the helical domains of both chaperones bind polypeptides but that hsp110 does not display this property in vitro. Another significant difference between hsp110 and grp170 was that the ABL mutant grp170 (unlike ABL hsp110) was capable of chaperoning denatured luciferase, while the AB mutant grp170 (like AB mutant hsp110) does not. This suggests that the inclusion of the acidic loop domain in grp170 has significance for binding of denatured protein by grp170. Notably, the L domain of grp170 is approximately 1.5 times as large as it is in hsp110 (approximately 150 amino acids in grp170 compared to 100 amino acids in hsp110).

Grp170 differs far more in its amino acid sequence from its cytoplasmic counterpart, hsp110, than grp78 differs from its cytoplasmic counterpart hsp70. Grp170 appears to be a multifunctional protein, involved in a number of ER associated functions, chaperoning immature secretory proteins, transport of peptides into the ER lumen, and transport of proteins from the ER lumen to the cytoplasm for degradation. Therefore, it is not surprising that this molecule displays multiple peptide binding domains. The presence of two strong peptide binding regions in such a large protein may facilitate the interactions and assembly of two substrate proteins. Further study will be required to clarify the role played by grp170 in vivo and to correlate this information with the structural and substrate binding characteristics of this large molecular mass ER resident molecular chaperone.

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