Functional Diversification and Specialization of Cytosolic 70-kDa Heat Shock Proteins

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A fundamental question in molecular evolution is how protein functional differentiation alters the ability of cells and organisms to cope with stress and survive. To answer this question we used two paralogous Hsp70s from mouse and explored whether these highly similar cytosolic molecular chaperones, which apart their temporal expression have been considered functionally interchangeable, are differentiated with respect to their lipid-binding function. We demonstrate that the two proteins bind to diverse lipids with different affinities and therefore are functionally specialized. The observed lipid-binding patterns may be related with the ability of both Hsp70s to induce cell death by binding to a particular plasma-membrane lipid, and the potential of only one of them to promote cell survival by binding to a specific lysosomal-membrane lipid. These observations reveal that two seemingly identical proteins differentially modulate cellular adaptation and survival by having acquired specialized functions via sequence divergence. Therefore, this study provides an evolutionary paradigm, where promiscuity, specificity, sub- and neo-functionalization orchestrate one of the most conserved systems in nature, the cellular stress-response.

A central question in molecular evolution is how sequence divergence of duplicated genes, either by mutations or domain shuffling, results in functional changes that enable organisms to adapt to their environment and thrive. At the cellular level the same essential question relates to the ability of cells and cellular systems to adapt and survive homeostatic imbalances due to stress.

To maintain homeostasis the cellular stress response (CSR) has evolved. The CSR is composed by several protein networks, which by interacting with each other and their ligands, e.g., proteins, lipids, or nucleic acids, regulate cell survival. Molecular chaperones are key regulators of the stress response system and alterations in their function have direct physiological consequences. The primary functions of several such proteins have been retained for several millions or billions of years.

The 70-kD heat shock proteins (Hsp70s) are one of the most compelling examples of proteins functioning in the CSR in which the primary function, protein folding and refolding, has been retained from bacteria to humans. Yet, in most eukaryotes certain Hsp70s acquired specialized functions that allowed cells and organisms to adapt, survive, and thrive in various environments. For example, several hsp70s are expressed constitutively in all cell types, others exhibit a tissue-specific constitutive expression pattern, and yet some others are expressed only in response to stress, e.g., heat-shock. Molecular chaperones are key regulators of the stress response system and alterations in their function have direct physiological consequences. The primary functions of several such proteins have been retained for several millions or billions of years.

Unlike other heat-shock proteins, such as the Hsp40s, which evolved specialized functions via sequence divergence and domain acquisition, Hsp70s have diverged exclusively via nucleotide mutations. Mutations at the coding regions of the genes resulted in specific amino acid changes that allowed the proteins to be targeted and retained to particular organelles, while mutations at the hsp70s’ promoter regions resulted in different expression patterns, e.g., constitutive or induced by stress. The coding region mutations seem to have occurred once or a few times during eukaryotic evolution, as the differently localized Hsp70s form deep phylogenetic clades dating back to the very first eukaryotes. In contrast, the promoters’ sequence changes, especially in the cytosolic hsp70s, have occurred multiple times during eukaryotic evolution, as it appears that heat-inducibility has evolved independently more than once in different phylogenetic lineages.
Figure 1 | Hsp70s have diverged very early in eukaryotic evolution and their expression pattern has changed multiple times. Hsp70 protein sequences from a few representative bacterial, archaebal, and eukaryotic species were used to generate trees with two different phylogenetic methods (NJ and ML). The numbers at the nodes are bootstrap values (>50; NJ/ML). The accession numbers of the sequences used are shown next to the protein and species name. CYT: cytosolic; ER: endoplasmic reticulum; CP: chloroplast; MT: mitochondrion. Fruit fly: Drosophila melanogaster; nematode: Caenorhabditis elegans; plant: Arabidopsis thaliana; yeast: Saccharomyces cerevisiae. Heat inducible genes are denoted with HI; genes with high basal expression and heat-inducible are depicted with *; high temperature heat inducible gene present in mammals, but absent in mouse is depicted with HI**.
Despite their temporal and spatial differentiation, all Hsp70s seem to perform the same chaperone functions. Therefore, it has been postulated that Hsp70s do not possess any functional specialization with respect to their protein clients5–11, with some notable exceptions, e.g., the cytosolic SSBs and the mitochondrial SSC2 in yeast, which function exclusively in nascent polypeptide folding12 and iron homeostasis13, respectively.

Yet, several reports have shown that specific human and mice Hsp70s localize at the plasma membrane (PM), associate with lipid-rafts, and bind to specific lipids14–28. Although the mechanism of these Hsp70s’ functions is less well characterized and understood compared to their protein chaperone functions, it has been demonstrated that the interaction of Hsp70s with membranes and lipids has direct physiological outcomes. These include activation of the immune system, viral entry, stabilization of the lysosomal membrane, microautophagy, trafficking of anandamide from the plasma membrane to internal cellular compartments, and promotion of cell apoptosis14–29. Still, it remains unknown how the lipid-binding function evolved, whether it is promiscuous or not, and if it functionally differentiates Hsp70s.

To answer some of these questions and shed light on the evolution of the Hsp70 lipid-binding functions we used two mammalian cytosolic proteins, HspA1A and HspA8, as a model system. These proteins combine five desirable characteristics that make them a suitable model to study the evolution of the Hsp70-lipid function. First, the genes coding for HspA1A and HspA8 are heat inducible and conserved in differentiating species, whether it is promiscuous or not, and if it functionally differentiates Hsp70.

To determine the spectrum of HspA1A and HspA8 potential lipid ligands we screened arrays of 36 lipids using the protein–lipid overlay (PLO) assay as implemented by Eschelon Biosciences. This initial screen showed that recombinant HspA1A binds to several anionic phospholipids, phosphatidylserine (PtdSer), phosphatidic acid (PtdOH), phosphatidylglycerol (PtdGro), cardiolipin (Ptd2Gro), and several phosphatidylinositolos (PtdIns), and a single sphingolipid sulfoylglycerolceramide (sulfatide; GalCer-1-sulfate) (Fig. 4a). To validate these initial results, get a semi-quantitative view of the lipid binding, and test whether both proteins bind to the same lipids we used an alternative form of the PLO assay by spotting different lipids and lipid amounts on nitrocellulose membranes. These assays confirmed the results of the first screen and strongly suggested that both HspA1A and HspA8 bind specifically to several anionic lipids (Fig. 4b).

Lipid-binding Screening. To determine the spectrum of HspA1A potential lipid ligands we screened arrays of 36 lipids using the protein–lipid overlay (PLO) assay as implemented by Eschelon Biosciences. This initial screen showed that recombinant HspA1A binds to several anionic phospholipids, phosphatidylserine (PtdSer), phosphatidic acid (PtdOH), phosphatidylglycerol (PtdGro), cardiolipin (Ptd2Gro), and several phosphatidylinositolos (PtdIns), and a single sphingolipid sulfoylglycerolceramide (sulfatide; GalCer-1-sulfate) (Fig. 4a). To validate these initial results, get a semi-quantitative view of the lipid binding, and test whether both proteins bind to the same lipids we used an alternative form of the PLO assay by spotting different lipids and lipid amounts on nitrocellulose membranes. These assays confirmed the results of the first screen and strongly suggested that both HspA1A and HspA8 bind specifically to several anionic lipids (Fig. 4b).

Lipid-binding Kinetics. To mechanistically characterize the lipid-binding properties of HspA1A and HspA8 we generated binding curves and determined binding kinetics (Fig. 6, Table 1, and Supplementary Tables S1 and S2). The results of these assays revealed that when the two proteins bind to the same lipid they have different: (a) affinities, values of apparent dissociation constants (Kd), (b) maximal binding (Bmax), and (c) in some cases, theoretical binding models. Furthermore, the significantly different dissociation values imply different lipid specificities, and the distinctive theoretical models suggest mechanistically altered lipid binding.
The affinity of HspA1A for BMP (K_d = 148 μM) is more than four times higher than that of HspA8 (K_d = 598 μM) for the same lipid (Fig. 6 and Table 1). The model that best describes the binding of both proteins to BMP is a modified Hill model (cooperative binding). The Hill coefficient, a, is smaller than 1 in the case of HspA1A, suggesting that the binding of one BMP molecule to the protein decreases its affinity for other BMP molecules (negatively cooperative binding). In contrast, the Hill coefficient is higher than 1 in the case of HspA1A (grey) and HspA8 (black) NBD region showing the overall structural similarities. The structural alignment was performed using DaliLite and the figures were generated with PyMol. The PDB codes of the structures used are: 3JXU for HspA1A and 3HSC for HspA8.

In the case of GalCer-1-sulfate, HspA1A and HspA8 show different affinities and maximal binding, with HspA8’s values (K_d = 85 μM; B_max = 73.6) being significantly higher than HspA1A’s (K_d = 111 μM; B_max = 56.7). Also, the theoretical models that best describe the interaction between the proteins and GalCer-1-sulfate suggest the presence of cooperative binding in the case of HspA1A and a non-cooperative binding (one site saturation) in the case of HspA8 (Supplementary Tables S1 and S2).

Lastly, both proteins bind to Ptd2Gro, a lipid found exclusively in the eukaryotic mitochondrial membrane and the bacterial PM, with comparable affinities and mechanism (Supplementary Tables S1 and S2).

**Discussion**

Three major observations summarize our results on the lipid-binding properties of the mouse HspA1A and HspA8. First, both proteins bind to several anionic lipids (Figs. 4 and 5). Second, the affinities of the interactions are relatively low (Table 1 and Table 1 and Supplementary Table S1). And third, both proteins bind to lipids with different affinities and specificities (Fig. 6, Table 1 and Supplementary Tables S1 and S2).
Figure 3 | Purified recombinant HspA1A and HspA8 are functional molecular chaperones. SDS-polyacrylamide gel electrophoresis of the His-tagged HspA1A and HspA8 proteins visualized with (a) Coomassie Blue staining and (b) Western blot using a rabbit polyclonal anti-His antibody.

The binding to anionic lipids could be the result of non-specific purely electrostatic interactions. However, this explanation seems highly unlikely because the amount of protein bound to a particular lipid does not increase as the charge increases. For example, the binding of HspA1A to Phosphatidylinositol 4-phosphate [PtdIns(4)P], which has a charge of −2, is much higher than to PtdIns(3,4,5)P3, which has a charge of −5. In addition to our data, other reports have shown that the interaction of HspA1A with particular lipids, e.g., PtdSer and PtdGro, does not depend solely on the charge of the lipid27. Therefore, the most plausible interpretation of our binding data is lipid specificity that depends on the chemical nature of the lipid head rather than its charge.

The observed affinities of the proteins for the lipids are rather low when compared to particular known lipid-binding domains, e.g., Pleckstrin Homology (PH)32,33. These apparent dissociation constants may be related to the in vitro nature of the assay used, which is based on the generation of artificial membranes lacking several of the components present in an actual cellular membrane and having different lipid composition, as well as technical limitations due to poor lipid solubility34.

Nevertheless, several examples in the literature demonstrate that low affinity interaction have specific and important biological functions, like the glucose transporters or hexokinase and glucokinase and their different affinity for glucose35–37. Additionally, despite the apparent low KD of Shiga toxin to its cellular receptor lipid globotriaosylceramide (GB3)38, the toxin enters the cells by specifically interacting with it. Also, although the affinity of Phospholipase C-ζ for particular phospholipid membranes is relatively low (molar partition coefficient K = 6 × 10^4 M^-1), the enzyme is anchored at the plasma membrane39. Additionally, the apparent dissociation constant of HspA2, a testis specific Hsp70, for the endogenous cannabinoid anandamide is ~4 μM30, yet HspA2 is a cytosolic carrier of this lipid. In these examples, low affinity interactions allow targeting and regulation of proteins and enzymes because only in the event of shifted equilibria specific binding would occur. Furthermore, the association of both HspA1A and HspA8 with PtdSer and the binding of the former protein with BMP have direct physiological effects4,27,29,35. Therefore, we infer that affinities with values close to the ones observed for HspA1A for PtdSer (KD = 14 μM) and BMP (KD = 148 μM) will be biologically important, while significantly lower affinities may not. Based on these inferences, which require experimental verification, we suggest that under the same physiological conditions: (a) only HspA1A binds to BMP and PtdOH, (b) only HspA8 binds to PtdGro, and c) both proteins bind to PtdSer, GalCer-1'-sulfate, and Ptd2Gro (Fig. 7).

The observed low affinities imply that high concentrations of particular lipids, e.g., PtdSer, BMP, or Ptd2Gro, or their combination are necessary for physiologically significant membrane binding of Hsp70s. Several of the lipids studied here, e.g., PtdSer, BMP,
Figure 4 | Qualitative protein-lipid overlay assay shows that recombinant HspA1A and HspA8 bind to several anionic lipids. (a) Echelon Lipid Strips using the HspA1A recombinant protein. (b) Membranes carrying serial dilutions of lipids (numbers on top in pmol) using both proteins. Each membrane was incubated with 12 nM of each protein for 1 h at room temperature and after extensive washes, the protein that remained on the membrane because of its interaction with lipids was visualized using an anti-His antibody. Representative blots of three independent experiments using different batches of purified protein are shown.

PtdOH, are particularly enriched in vesicular bodies, recycling and early endosomes, and lysosomes.\(^{40-46}\). For example, the concentration of BMP in the late endosome is \(\sim 15\) mol % of the total lipid content of the organelle and can comprise as much as 70 mol % of the lipid composition of the intraendosomal vesicles.\(^{42,47}\). Furthermore, the concentration, localization, and saturation levels of particular lipids are altered in particular cell types (e.g., red-blood cells\(^{48,49}\)), or because of stress (e.g., heat-shock), disease (e.g., cancer, Gaucher, Niemann-Pick), or during apoptosis\(^{29,40,50-56}\). These changes in membrane topography and lipid/protein ratios may facilitate the interaction of Hsp70s with lipids. The fact that Hsp70s have been observed to interact with lipids and membranes mainly in stressed, pre-apoptotic, or cancerous cells\(^{13,17,20,27,29,57-61}\) supports the above notion. Therefore, we predict that Hsp70s bind to lipids in particularly stressful conditions when enough lipid is present in specific membranes.

We further infer that the observed lipid-binding quantitative values may correlate with specific biologically important properties of HspA1A and HspA8. For example, both chaperones have been shown to promote cell death by binding externalized PtdSer at the plasma membrane of pre-apoptotic and cancerous cells\(^{13,17,20,27}\) and our results reveal that both HspA1A and HspA8 bind PtdSer with comparable high affinities (Table 1 and Fig. 7). Additionally, the relatively high affinity of HspA8 for PtdSer may be related with its ability to interact with PtdSer at the endosomal membrane and mediate microautophagy.\(^{29}\). Given the similar affinities of the two proteins for PtdSer, however, it is not clear why HspA1A was not observed to perform the same function. We speculate that HspA1A, which is inducible and exists in minimal amounts in non-stressed cells, is not present in substantial amounts in the cell during the events of microautophagy. Furthermore, HspA1A binds to BMP and rescues the lysosomal membrane, while disruption of this interaction results in lysosomal membrane permeabilization\(^{29}\). In contrast, HspA8 fails to rescue the lysosomal membrane.\(^{29}\) Given the very low affinity of HspA8 for BMP (Table 1 and Fig. 7), we suggest that this protein may not bind BMP under physiological conditions. This notion, which warrants experimental validation, might explain why HspA8 does not rescue lysosomes while HspA1A does\(^{29}\).

Promotion of cell-death, microautophagy, and lysosomal rescue are important biological processes that can be explained by the presence or absence of a specific Hsp70-lipid interaction. Furthermore, basic biological processes such as cell choice between survival and death, immune response, neurotransmission, energy homeostasis, and reproduction are controlled by the intracellular trafficking of endocannabinoids, which, is mediated by HspA2 among others\(^{62}\). We speculate that the binding of Hsp70s to lipids such as PtdOH, GalCer-I\(^{-}\)-sulfate, and Ptd2Gro (Fig. 7) have physiological implications. Our overarching hypothesis, which is founded on the observed Hsp70-lipid interactions and the chemical properties of the lipids as well as their known physiological roles, states that lipid binding targets the chaperones to specific cellular sites, where, besides stabilizing proteins, they also stabilize and/or alter membranes. Hence, the Hsp70-lipid interaction provides these promiscuous protein chaperones efficient specificity to localize and function at particular membranes during cellular stress.
The specific binding of both chaperones to particular lipids suggests that lipid binding is a conserved function of HspA1A and HspA8 since their appearance (Fig. 1). We propose that the common ancestor of these proteins would also bind to particular lipids. Although we do not know which these lipids were, we speculate that PtdSer, which both proteins bind (Table 1 and Fig. 7), is one of the lipids that their common ancestor bound. Moreover, both proteins bind to GalCer-I3-sulfate and Ptd2Gro. Taking into account that several bacterial Hsp70 homologs (DnaKs) bind to GalCer-I3-sulfate24, and Ptd2Gro is a lipid predominantly found in bacteria and the bacterial symbionts of eukaryotes, we reason that lipid-binding is an ancient function of Hsp70s conserved for several billion years. Yet, because we do not know which other lipids the bacterial Hsp70s bind, we cannot define precisely the evolution of their lipid-binding function. Furthermore, the observed Hsp70-lipid binding differences suggest that the ancient ability of these proteins has evolved. Still, it is unclear whether these changes are the result of sub- or neo-functionalization or a combination of both.

Nonetheless, our data combined with the spatially confined and differential expression of Hsp70s allow us to provide a speculative scenario for the evolution of the Hsp70-lipid binding function. Our scenario suggests a combination of sub- and neo-functionalization64–67 and it is based on two assumptions. First, the ancestral hsp70 genes were expressed under both normal and stress conditions like the bacterial hsp70, dnaK (Fig. 1)68. And second, compared to HspA1A, HspA8 is functionally and evolutionarily closer to their common ancestor, because: (a) hspA8 has clear orthologs in all vertebrates (Fig. 1), while hspA1A does not6; (b) hspA1A is heat inducible and inducibility has evolved independently several times (Fig. 1); and (c), HspA8 binds to PtdGro (Fig. 7), the predominant bacterial lipid, while HspA1A does not.

According to our scenario, the coding sequence of the ancestral hsp70 genes diverged via nucleotide substitutions very early during evolution of the eukaryotic ancestor. The specific binding of both chaperones to particular lipids suggests that lipid binding is a conserved function of HspA1A and HspA8 since their appearance (Fig. 1). We propose that the common ancestor of these proteins would also bind to particular lipids. Although we do not know which these lipids were, we speculate that PtdSer, which both proteins bind (Table 1 and Fig. 7), is one of the lipids that their common ancestor bound. Moreover, both proteins bind to GalCer-I3-sulfate and Ptd2Gro. Taking into account that several bacterial Hsp70 homologs (DnaKs) bind to GalCer-I3-sulfate24, and Ptd2Gro is a lipid predominantly found in bacteria and the bacterial symbionts of eukaryotes, we reason that lipid-binding is an ancient function of Hsp70s conserved for several billion years. Yet, because we do not know which other lipids the bacterial Hsp70s bind, we cannot define precisely the evolution of their lipid-binding function. Furthermore, the observed Hsp70-lipid binding differences suggest that the ancient ability of these proteins has evolved. Still, it is unclear whether these changes are the result of sub- or neo-functionalization or a combination of both.

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eukaryotic evolution, because spatially confined Hsp70s form deep phylogenetic clades (Fig. 1). This relatively rapid sequence divergence, sub-functionalization introduced signal peptides and retention motifs that targeted and limited the proteins at particular subcellular compartments resulting in specialized spatial expression patterns. Additionally, during the course of their evolution hsp70 genes acquired neutral mutations in their promoter regions; some copies retained part of the ancestral expression pattern whereas others did not resulting in either constitutively expressed or heat-induced genes (Fig. 1).

Despite the divergence in both coding and non-coding gene sequences, the amino acid sites responsible for the protein chaperone function were retained by strong purifying selection. However, the sites responsible for lipid-binding mutated, reached fixation, and ensued biochemical specialization and functional differentiation. Such sequence divergence most probably occurred with the evolution of membrane lipid composition, which must have affected cell survival. Functionalization was followed by prolonged neo-functionalization resulting in binding to new lipid ligands, for example BMP. The neo-functionalizing mutations were most probably adaptive because they have affected cell survival.

The differential lipid-binding profiles of the highly conserved and almost identical molecular chaperones HspA1A and HspA8 reveal that sequence divergence via single amino acid replacements led to highly specialized functions, which directly affected cellular homeostasis. In this report we define an evolutionary path, where promiscuous functions to specific subcellular compartments resulting in specialized spatial expression are depicted with grey filled boxes. Significantly lower affinities (probably the protein does not bind the lipid in a cell) are depicted with black filled boxes.

**Table 1** Binding kinetics of HspA1A (A1A) and HspA8 (A8) to PtdSer and BMP.

|         | PtdSer | BMP |
|---------|--------|-----|
| HspA1A  |        |     |
| A1A     | 14 ± 1.9 | 598 ± 73.2 |
| A8      | 72 ± 8.3  |         |
| HspA8   |        |     |
| A1A     | 148 ± 19.1 |       |
| A8      | 598 ± 73.2 |       |

Kd (apparent dissociation constant), μM; Bmax (maximal binding); % protein bound; a (Hill coefficient); B0, initial binding; Ns, non-specific binding.

**Methods**

**Lipids, Chemicals, and Reagents.** Phosphatidylcholine [PtdCho; 1,2-dipalmitoyl-sn-glycero-3-phosphocholine], Phosphatidylethanolamine [PtdEtn; 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine], Phosphatidylserine [PtdSer; 1,2-dipalmitoyl-sn-glycerol-3-phosphoserine (sodium salt)], Phosphatidic acid [PtdOH; 1,2-dipalmitoyl-sn-glycerol-3-phosphate (sodium salt)], Phosphatidylglycerol [PtdGro; 1,2-dipalmitoyl-sn-glycerol-3-phosphoglycerol (sodium salt)], Phosphatidylinositol [PtdIns; 1,2-dipalmitoyl-sn-glycerol-3-phospho-(1’-myo-inositol) (ammonium salt)], Phosphatidylinositol 4-phosphate [PtdIns(4)P; 1,2-dioleoyl-sn-glycerol-3-phospho-(1’-myo-inositol-4’-phosphate) (ammonium salt)], Phosphatidylinositol 3,5-bis-phosphate [PtdIns(3,5)P2; 1,2-dioleoyl-sn-glycerol-3-phospho-(1’-myo-inositol-3’,5’-bisphosphate) (ammonium salt)], and Phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P3; 1,2-dioleoyl-sn-glycerol-3-phospho-(1’-myo-inositol-3’,4’,5’-trisphosphate) (ammonium salt)]. Cardiolipin [PtdEtn; 1,1’,2’,2’-tetramyristoyl cardiolipin (ammonium salt)], Galactosyl Ceramide [GalCer; D-galactosyl-b-D-1’,1’-N-palmito-D-erythro-sphingosine], Sulfatide [GalCer-D-sulfate; 3-O-Sulfo-D-Galactosyl-b-D-1’,1’-N-Lignoceryl-D-erythro-sphingosine (ammonium salt)], bis(monoacylglycerol)phosphatidylserine (sn-3-oleoyl-2-hydroxy)-glycerol-1-phospho-sn-3’-(1’-oleoyl-2’-hydroxy)-glycerol (ammonium salt)], and Cholesterol [Ch; (3’)-cholest-5-en-3-ol)] were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) and Echelon Biosciences Inc. Salt Lake City, UT). Other common chemicals and reagents, e.g., antibiotics, buffers, and growth media, were obtained from Fisher Scientific or Sigma-Aldrich (St. Louis, MO).

**Protein sequence analysis and phylogeny.** Protein sequences were collected from the National Center for Biotechnology Information (NCBI) protein databank using either keyword or protein-BLAST searches with the human HspA1A and HspA8 as queries and default parameters. The sequences were aligned with MAFFT using the E-INS-i strategy and default parameters, and manually corrected with BioEdit. Maximum-likelihood (ML) was used to find the best model of evolution (MEGA 6). The dataset consisted of 25 sequences using the neighbor-joining (NJ) and ML algorithms as implemented in MEGA 6. One thousand bootstrap pseudo-replicates were used to test the reliability of the inferred trees. All positions containing gaps were eliminated and there was a total of 559 positions in the final dataset.

**Generation of Recombinant DNA Clones.** The mouse cDNA clones containing the hspA1A and hspA8 gene sequences, accession numbers BC057482 and BC089457 respectively, were purchased from Open Biosystems (GE Dharmacon). Clones corresponding to the HspA1A and HspA8 full-length gene were generated by the polymerase chain reaction (PCR) method using specific 5’- and 3’-primer sets. The primer sequences used as well as the restriction enzymes (Ndel and Xhol; underlined nucleotides) that were incorporated for directional cloning of the genes were: CCAGACATATACGACATCGTCAATCTGAATGCT for hspA1A and CCAGACATATACGACATCGTCAATCTGAATGCT for hspA8.

The amplified DNA fragments were then cloned into the protein expression vector pET-22b+ (Novagen) using the Rapid DNA Ligation Kit (Roche) following the manufacturer’s protocol. The ligation mixtures were later transformed in E. coli strain DH5α cells (Life Technologies), the positive colonies were verified by PCR, and the intact open reading frames were verified by DNA sequencing.

**Generation and Purification of Recombinant Proteins.** Purified plasmid DNA of sequence-verified recombinant clones was subsequently transformed into E. coli BL21 (DE3) E. coli cells (Life Technologies). A single colony was then added to 15 mL of Luria-Bertani (LB) broth with Ampicillin (100 μg/mL) and grown until an OD of between 0.8 and 1.0 was reached. Recombinant protein production was induced using 1 mM (final concentration) of Isopropl β-D-1-thiogalactopyranoside (IPTG) at 25 C for 14–16 h. The cultures were pelleted by centrifugation and the cells were lysed in a lysis buffer containing 50 mM sodium phosphate, pH 7.4, and 300 mM sodium chloride. During lysis, Phenylmethlysulfonyl fluoride (PMSF) (1 mM), lysozyme (0.5 mg/mL), and Triton X-1% (1%) were added, and the lysates were sonicated until optically clear. After sonication, the lysates were rotated at 4°C for 30 min and were centrifuged at 10,000 × g for 5’. The supernatant, containing the soluble Hsp70 proteins, was equilibrated in glutaraldehyde (2%) and then incubated with the same buffer and rotated, at 4°C for 1 h. The samples were then centrifuged at 700 × g for 2 min and the beads were washed 3× with the same buffer to remove proteins that did not interact with the cobalt beads. Finally, the recombinant proteins were eluted from the beads by incubation with equal volume of lysis buffer containing 150 mM imidazole. The elutions were then dialyzed extensively against a 25 mM Tris-HCl or 25 mM HEPES, pH 7.4, buffer using Amicon Ultra centrifugal filters.

The protein concentration was determined using the Coomassie Blue Plus Protein Assay Reagent (Pierce) following the protocol supplied by the manufacturer. The
concentration (μg/ml) values were transformed to moles/ml by dividing them with the protein molecular weight. Protein purity was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were detected by staining with Simply Blue Safe Stain (Life Sciences). For Western blotting, separated proteins were transferred to nitrocellulose (Protran; Whatman) and blocked with 5% milk powder, in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween 20 (TBST) for 1 h at room temperature. Western blots were probed with 1:1000 polyclonal HspA1A (Thermo Scientific; 2000 : 2000 in TBST) overnight at 4°C. The secondary antibody, peroxidase-conjugated goat anti-rabbit immunoglobulin (Thermo Scientific: 1 : 10000 in TBST), was incubated with the nitrocellulose for 1 h at room temperature. Bound antibody was visualized with the Pierce ECL Western Blotting Substrate (Pierce). All gels and signals were collected using the Omega Lum C system from Aplegen.

Protein Activity Tests. To test whether the recombinant proteins were functional molecular chaperones it was determined whether they (i) suppress protein aggregation, (ii) refold denatured proteins, and (iii) hydrolyze ATP. All experiments were repeated at least three times using different batches of protein. (i) The aggregation assay included the chemical denaturation of lysozyme by incubating 10 mg/ml of lysozyme at 40°C for 2 hours into a buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA. The denatured enzyme was then diluted 100 fold in a non-denaturing buffer (50 mM Tris-HCl, pH 8.0, 80 mM NaCl, and 1 mM EDTA), which resulted in lysozyme precipitation. The aggregation rate was then monitored by measuring the increase in light scattering (turbidity) as a result of protein aggregation spectrophotometrically at 340 nm every minute for 15 min. Addition of 2 μM HspA1A and HspA8 prevented the turbidity of the solution to increase, showing that these recombinant proteins prevent protein aggregation. Control reactions containing no chaperones were used. (ii) The β-galactosidase refolding assay used chemically denatured β-galactosidase and measured the recovery of the enzyme’s activity by hydrolysis of the ortho-Nitrophenyl-β-galactoside relative to the activity of the native enzyme after addition of ATP and recombinant Hsp70s. BSA and reactions with no enzyme were used as negative controls. Specifically, β-galactosidase was added to either Glycylglycine (native buffer) or to unfolding buffer (25 mM HEPES, pH 7.4, 5 mM MgCl₂, 50 mM KCl, 2 mM ATP, 10 mM DTT). The enzyme was incubated at 30°C for 30 min. The reactions contained the control protein BSA (3.2 μM) or Hsp70 (2 μM) and β-galactosidase (native or denatured) to 1 μM each were incubated at 37°C for the duration of the experiment. Samples were taken from each reaction every 30 minute for a total of 240 min, added to ONPG, and incubated at 37°C for 15 min. These enzymatic reactions were stopped by the addition of 0.5 M sodium carbonate. The absorbance was read at 450 nm and the percent activity of the β-galactosidase was determined using the following equation: % activity = 100*(test absorbance/native-absorbance). Percent activity was plotted versus time to compare the amount of β-galactosidase that recovered its function, which correlates with the amount of β-galactosidase refolded by the chaperone. (iii) The ATPase assay used is a colorimetric assay that measures the amount of free inorganic phosphate. This assay was performed by incubating 1 μM of recombinant Hsp70 with 4 mM ATP at 37°C for 120 minutes and the release of inorganic phosphate (Pi) was quantified every 30 min using the colorimetric assay QuantChrom™ ATPase/GTase Assay kit (BioAssay Systems). To determine the amount of Pi released a standard curve was generated by measuring the absorbance produced by known Pi concentrations. Controls that contained no chaperone or BSA were also used to account for spontaneous ATP hydrolysis. The final amounts of Pi released were calculated by subtracting the control values from each sample.

Lipid Binding Assays. The verified for functionality proteins were then tested for their ability to bind to lipids using the well-established protein-lipid overlay (PLO) and lipid vesicle sedimentation (LVS) assays. The PLO assay served as a first screen to determine the spectrum of lipids that the protein interacts with. For PLO assays, 5 μg of recombinant Hsp70s were incubated with 0.1 μg of liposomes. To identify lipid ligands, it contains a fixed amount of lipids and a limited array of lipids. In the second PLO assay, 2 μg of recombinant Hsp70s were incubated with 0.1 μg of liposomes. Control lipids are those in cellular membranes. Therefore, we used proteins that do not precipitate and several control-lipids. Precipitation is not promoted by the presence of the lipid due to non-specific binding. Lipid mixtures tested and their mol/mol ratios (given in parenthesis) were: PtdCho:PtdIns (95:5), PtdCho:PtdIns(4)P (95:5), PtdCho:PtdIns(3)P2 (95:5), PtdCho:PtdIns(PtdSer) (95:5), PtdCho:PtdIns(4)P5 (95:5). PtdCho:PtdIns4P (95:5). PtdCho:PtdIns(3)P2 (95:5). PtdCho:PtdIns4P (95:5). PtdCho:GlcCer (95:5). PtdCho:GlcCer-Tf-sulfate (90:10). PtdCho:BMP (70:30). These mixtures were dried under vacuum for approximately 40 min and then were hydrated at room temperature for 1 hour in a buffer containing 25 mM HEPES, pH 7.4, and 100 mM NaCl (HBS) and vortexed frequently. Within 30 min of hydration, the samples were probe-sonicated for 10 x 1 second bursts. After 1 hour hydration, multilayered vesicles were formed. To generate the final uniform population of small, unilamellar vesicles, the samples were subjected to five cycles of freezing in liquid nitrogen and thawing in a bath sonicator (45°C, 10 minute sonicits) until optically clear. The binding reactions contained a fixed concentration (1 μM) of Hsp70 in a total reaction volume of 100 μl. A specific concentration of lipid vesicles was added and the reactions were incubated at 30°C for 30 min. After the incubation period, the samples were transferred to ultracentrifuge tubes and ultracentrifuged at 166,000 × g at 25°C for 40 min. After centrifugation, the supernatant was removed and saved in microcentrifuge tubes. The pellets were then resuspended in equal volume of HBS. Equal volumes of supernatant and pellet fractions, which contained unbound and bound to liposomes proteins, respectively, were used to graph all lipid vesicle sedimentation assay data and to fit the binding data to various equations corresponding to specific binding models.

Binding affinities. To determine the affinity of HspA1A and HspA8 to various lipids the LVS assay was performed using increasing concentrations of vesicles (0.1–4 mM) and a fixed concentration (1 μM) of protein. The reactions were incubated and processed exactly as described above. All experiments were repeated with at least three different batches of protein. SigmaPlot (version 10.0, Systat Software Inc) was used to graph all lipid vesicle sedimentation assay data and to fit the binding data to various equations corresponding to specific binding models.

Statistical tests. Non-linear regression analysis as implemented in SigmaPlot (version 10.0, Systat Software Inc) was used to determine the goodness of fit of the data to a particular theoretical binding model. The same program was also used to calculate mean binding and standard deviations. Statistical significance was determined by an unpaired t-test. A P value < 0.05 was considered statistically significant.
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**Author contributions**

C.M. and N.N. designed the study. C.M., M.C.S., F.S. and N.N. performed the experiments. C.M., D.C. and N.N. analyzed the data and performed statistical analysis. C.M., D.C. and N.N. wrote the manuscript. The contents are the responsibility of the authors and do not necessarily reflect the views of USAID or the United States Government.

**Additional information**

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