Title

Interaction of heat shock protein 70 with membranes depends on the lipid environment

Journal

Cell Stress and Chaperones, 19(6)

ISSN

1355-8145

Authors

Armijo, Gabrielle
Okerblom, Jonathan
Cauvi, David M
et al.

Publication Date

2014-11-01

DOI

10.1007/s12192-014-0511-x

Peer reviewed

Cell Stress and Chaperones (2014) 19:877–886
DOI 10.1007/s12192-014-0511-x

ORIGINAL PAPER

Interaction of heat shock protein 70 with membranes depends on the lipid environment

Gabrielle Armijo · Jonathan Okerblom · David M. Cauvi · Victor Lopez · Diana E. Schlamadinger · Judy Kim · Nelson Arispe · Antonio De Maio

Received: 28 February 2014 / Revised: 3 April 2014 / Accepted: 4 April 2014 / Published online: 1 May 2014

Abstract Heat shock proteins (hsp) are well recognized for their protein folding activity. Additionally, hsp expression is enhanced during stress conditions to preserve cellular homeostasis. Hsp are also detected outside cells, released by an active mechanism independent of cell death. Extracellular hsp appear to act as signaling molecules as part of a systemic response to stress. Extracellular hsp do not contain a consensus signal for their secretion via the classical ER-Golgi compartment. Therefore, they are likely exported by an alternative mechanism requiring translocation across the plasma membrane. Since Hsp70, the major inducible hsp, has been detected on surface of stressed cells, we propose that membrane interaction is the first step in the export process. The question that emerges is how does this charged cytosolic protein interact with lipid membranes? Prior studies have shown that Hsp70 formed ion conductance pathways within artificial lipid bilayers. These early observations have been extended herewith using a liposome insertion assay. We showed that Hsp70 selectively interacted with negatively charged phospholipids, particularly phosphatidyl serine (PS), within liposomes, which was followed by insertion into the lipid bilayer, forming high-molecular weight oligomers. Hsp70 displayed a preference for less fluid lipid environments and the region embedded into the lipid membrane was mapped toward the C-terminus end of the molecule. The results from our studies provide evidence of an unexpected ability of a large, charged protein to become inserted into a lipid membrane. This observation provides a new paradigm for the interaction of proteins with lipid environments. In addition, it may explain the export mechanism of an increasing number of proteins that lack the consensus secretory signals.

Keywords Liposomes · Hsp70 · Membranes · Stress

Gabrielle Armijo, Jonathan Okerblom, and David M. Cauvi contributed equally to this project.

G. Armijo · J. Okerblom · V. Lopez
Initiative for Maximizing Student Development (IMSD) Program, University of California San Diego, La Jolla, CA, USA

D. M. Cauvi
Department of Surgery, School of Medicine, La Jolla, CA, USA

D. E. Schlamadinger · J. Kim
Department of Chemistry and Biochemistry, University of California San Diego, La Jolla, CA, USA

N. Arispe
Uniformed Services University, Bethesda, MD, USA

A. De Maio
Center for Investigations of Health and Education Disparities, University of California San Diego, La Jolla, CA, USA

A. De Maio (✉)
Departments of Surgery and Neuroscience, School of Medicine, University of California San Diego, 9500 Gilman Drive, #0739, La Jolla 92093-0739, CA, USA
e-mail: ademaio@ucsd.edu

Introduction

Heat shock proteins (hsp) comprise a family of evolutionarily conserved polypeptides that participate in many cellular processes, particularly in the folding of newly synthesized polypeptides, which has inspired their designation as molecular chaperones. In addition, their expression is increased during stress conditions, and their function is vital for the recovery of cells after the insult. Moreover, they confer protection from subsequent stresses, which has been coined stress tolerance (De Maio 1999; Hartl and Hayer-Hartl 2009). Hsp are mainly localized in subcellular compartments, including the cytosol, endoplasmic reticulum, and mitochondria, where they display their chaperone function. In addition, hsp have been found outside cells where they appear to play a different function

from their chaperone activity. Extracellular hsp have been proposed to act as signaling molecules that activate a systemic response to stress, in particular, at the level of the immune system (De Maio 2011). The presence of several extracellular hsp has been associated with an array of pathological conditions (De Maio 2011; De Maio and Vazquez 2013). Hsp are secreted by an active mechanism in living cells, which could not be blocked by inhibitors of the classical ER-Golgi pathway (Hightower and Guidon 1989; Hunter-Lavin et al. 2004). Since cytosolic hsp that are secreted do not contain a consensus secretory signal that allows them to be transported through the ER-Golgi compartment, they could be exported by an alternative mechanism coined the non-classical secretory pathway. Several signaling molecules such as interleukin 1 and high-mobility group box 1 are exported via this alternative pathway (Nickel and Seedorf 2008).

The biggest challenge for the translocation of a protein from the cytosol to the extracellular environment is the crossing through the plasma membrane, which results in a less favorable change in free energy (Wimley et al. 1998). We have previously shown that the major inducible form of the hsp family, Hsp70, as well as its constitutive homologue, Hsc70, can be incorporated into artificial lipid bilayers and open ion conductance pathways (Arispe and De Maio 2000; Vega et al. 2008). In addition, Hsp70 has been shown to be present on the surface of transformed or stressed cells (Multhoff and Hightower 1996; De Maio 2011). Therefore, we have proposed that the export process of Hsp70 involves its initial insertion into the plasma membrane (De Maio 2011). Then, the protein, still inserted into the lipid bilayer, is released within extracellular vesicles (ECV) (De Maio 2011; Vega et al. 2008). ECV containing Hsp70 have been shown to activate a robust response in cells of the immune system (Vega et al. 2008). The question that emerges is how does a protein within the cytosol with a high number of charged amino acids and no major hydrophobic domains become inserted into the plasma membrane, which contradicts any thermodynamic prediction? To address this question, we developed a simple assay to investigate the mechanism of Hsp70 membrane interaction. We found that, indeed, Hsp70 incorporates spontaneously into the lipid bilayer of negatively charged lipids. Upon insertion, the protein oligomerizes within the membrane. This novel observation provides a new paradigm for the interaction of proteins with lipid environments.

Materials and methods

Liposome preparation and incorporation of Hsp70

Liposomes were formed by resuspending the dried lipid film (400 μg, Avanti Polar Lipids, Alabaster, AL) in 50-mM Tris Buffer pH 7.4 (120 μl), and vortexed every 5 min for 30 min. The preparation was extruded through a 100-nm membrane filter (15 passages). Thereafter, liposomes were incubated with recombinant Hsp70 (ADI-ESP-555, HSPA1A, expressed in *Escherichia coli*, Enzo Life Sciences, Farmingdale, NY) with 50-mM Tris Buffer pH 7.4 for 30 min at 25 °C at a ratio of 400 μg lipids per 1 μg of protein. Hsp70-containing liposomes were centrifuged at 100,000×*g* for 40 min at 4 °C and washed once with a sodium carbonate solution (pH 11.5). The final pellet after centrifugation (Hsp70-containing liposomes) was resuspended in lithium dodecyl sulfate (LDS) sample buffer and boiled for 8 min. The samples were resolved by LDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane. The presence of Hsp70 on the blot was detected by monoclonal anti-Hsp70 antibodies (SPA810, Enzo Life Sciences), and HRP-conjugated goat-anti-mouse secondary antibodies (Thermo Scientific, Rockford, IL). After incubation with the primary and secondary antibodies, the nitrocellulose membrane was visualized by chemiluminescence.

Methods for fluorophore encapsulation and leakage

Dye leakage assays were performed as previously described (Gable et al. 2009) with minor modifications. Dried lipids were resuspended in 50-mM Tris–HCl pH 7.4 containing 50-mM 8-aminonaphthalene-1,3,6-trisulfonate (ANTS) as fluorophore and 50-mM quencher *p*-xylenebis (pyridinium) bromide (DPX) as quencher, and vortexed every 5 min for 30 min. Vesicles were then extruded through a 200-nm filter 15 times. Dye-containing vesicle solutions were then passed through a gravity-driven desalting column (10-DG Bio-Rad, Hercules, CA) to separate free dye from vesicles. Hsp70 was then incubated with vesicles in 50-mM Tris buffer pH 7.4 for 30 min at room temperature unless otherwise specified. Spectra were then obtained on a Jobin Yvon HORIBA FluoroLog-3 Model FL3-11 spectrofluorometer. The excitation wavelength was 386 nm, and the bandpass for entrance and exit slits was set to 4 nm. To determine 100 % dye leakage, vesicles were incubated with 0.2 % Triton X-100 for 30 min before acquisition.

Results

Hsp70 specifically interacts with POPS liposomes

We have previously shown that Hsp70 is incorporated into artificial lipid bilayer opening ion conductance pathways (Arispe and De Maio 2000; Vega et al. 2008). To investigate further the interaction of Hsp70 with lipid membranes, we used a liposome incorporation assay. Pure recombinant Hsp70 was incubated with unilamellar liposomes made of phosphatidylserine (POPS) or phosphatidylcholine (POPC) for 30 min at 25 °C. Liposomes and proteins in the incubation

medium were separated by high-speed centrifugations. The pellets containing the liposomes were washed by repeating the centrifugation step, and the presence of Hsp70 in the liposome fraction (pellet) was detected by Western blotting. We found the presence of Hsp70 within POPS liposomes, but not on POPC liposomes (Fig. 1a). As a control, we used another member of the hsp family, Hsp90. This protein was not incorporated into the liposomes (POPS or POPC), suggesting that the presence of Hsp70 within the liposome pellet was not due to an artifact of the sedimentation process (Fig. 1a). To demonstrate that, indeed, Hsp70 was incorporated into the liposome membrane and was not just an external association with the vesicles, liposomes in which Hsp70 was already incorporated as described above were washed with buffers at low or high pH (2 or 11.5). This procedure did not result in the removal of the protein from the liposomes (Fig. 1b). Based on this result, a washing step at pH 11.5 (Na_2CO_3) was included in the subsequent analysis. Liposomes containing Hsp70 also withstood sonication treatment, suggesting that Hsp70 is not in the liposome lumen, but rather incorporated within the lipid bilayer (Fig. 1c). The only treatment that resulted in the release of Hsp70 from the liposomes was solubilization with Triton X-100 (Fig. 1d). In addition, we tested the incorporation of Hsp70 using other lipids. Hsp70 was not significantly

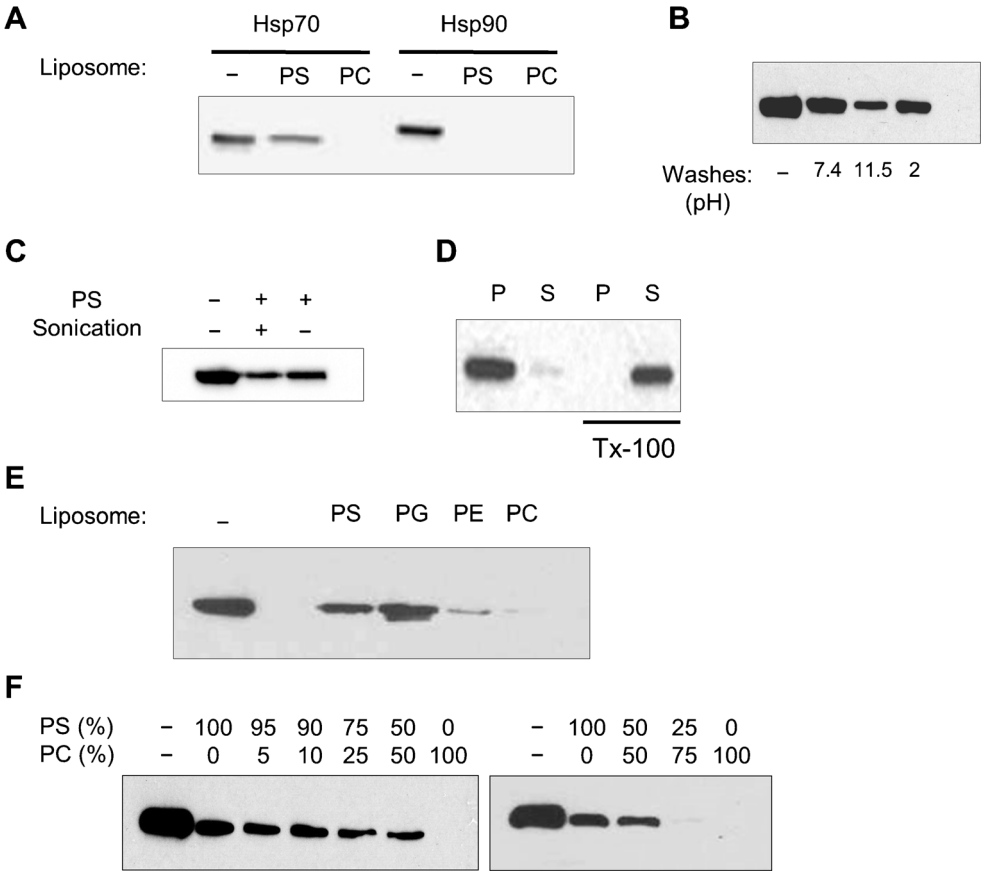

Fig. 1 Hsp70 is incorporated into the membrane of liposomes. **a** Pure recombinant Hsp70 (1 μg) or Hsp90 (1 μg) were incubated with POPS (PS) or POPC (PC) liposomes (400 μg) in 50-mM Tris buffer, pH 7.4 for 30 min at 25 °C. At the end of the incubation period, liposomes were centrifuged at 100,000×*g* for 40 min at 4 °C. The pellet was resuspended and analyzed by Western blotting using a monoclonal antibody against Hsp70 (SPA810, Enzo Life Sciences) or against Hsp90 (Enzo Life Sciences). A sample of Hsp70 (1 μg) or Hsp90 (1 μg) was included as a control in the Western blot. **b** POPS liposomes containing Hsp70 were incubated with 50-mM Tris buffer, pH 7.4, 100-mM Na_2CO_3, pH 11.5 or 100-mM glycine, pH 2 for 30 min at 4 °C and centrifuged at 100,000×*g* for 40 min at 4 °C, and the pellet was resuspended and analyzed by Western blotting. A sample of Hsp70 (1 μg) was included within the Western blot. **c** POPS liposomes containing Hsp70 were sonicated for 1 s 10 times or not, centrifuged at 100,000×*g* for 40 min at 4 °C, and the pellet was resuspended and analyzed by Western blotting. A sample of Hsp70 (1 μg) was included within the Western blot. **d** POPS liposomes containing Hsp70 were washed with 100-mM Na_2CO_3, pH 11.5 and treated or not with Triton X-100 (Tx100, 1 % final concentration) for 30 min at 4 °C. Samples were centrifuged, and both pellets (P) and supernatants (S) were analyzed by Western blotting. **e** Recombinant Hsp70 (1 μg) was incubated with POPS (PS), POPG (PG), POPE (PE), or POPC (PC) liposomes (400 μg) in 50-mM Tris buffer, pH 7.4, for 30 min at 25 °C. Liposomes were centrifuged at 100,000×*g* for 40 min at 4 °C, washed with 100-mM Na_2CO_3, pH 11.5, and analyzed by Western blotting. A sample of Hsp70 (1 μg) was included within the Western blot. **f** Liposomes made of a different mixture of POPS (PS) and POPC (PC) as indicated in the figure, maintaining the total concentration of lipid constant (400 μg). Pure recombinant Hsp70 (1 μg) was incubated with liposomes in 50-mM Tris buffer, pH 7.4 for 30 min at 25 °C. At the end of the incubation period, liposomes were centrifuged at 100,000×*g* for 40 min at 4 °C and were washed with 100 mM Na_2CO_3, pH 11.5. The pellet was resuspended and analyzed by Western blotting. As a control, a sample of Hsp70 (1 μg) was included within the Western blot

incorporated into phosphoethalonamine (POPE) liposomes, but it was inserted into phosphatidylglycerol (POPG) liposomes, which is another anionic phospholipid like POPS (Fig. 1e). Since Hsp70 is incorporated into POPS liposomes, but not into POPC liposomes, we studied the incorporation of the protein into a mixture of these two lipids. The incorporation of Hsp70 into liposomes was proportionally reduced by decreasing the content of POPS in the liposomes while maintaining the same total amount of lipids within the liposome. Further substitution of POPS with POPC above 50 % resulted in complete inhibition of Hsp70 incorporation into the liposomes (Fig. 1f), suggesting that there is a minimum number of phosphatidyl serine (PS) moieties necessary for Hsp70 incorporation into membranes. The incorporation of Hsp70 into liposomes was also studied using liposomes encapsulated with fluorophore (ANTS) and a quencher (DPX). The incorporation of Hsp70 resulted in a disturbance of the membrane, which allowed for the leakage of some fluorophore that, after separating from the quencher (DPX), resulted in fluorescent emission. As a positive control, liposomes were incubated with Triton X-100, which resulted in total disruption of the liposomes. The incorporation of Hsp70 into the liposome caused a disturbance of the membrane that allowed the leakage of the fluorescent dye, which did not increase dramatically over the incubation time (Fig. 2a), suggesting that after the initial membrane disturbance, Hsp70 association with the lipid bilayer is stable, as previously reported, regarding the ion channel activity (Vega et al. 2008; Arispe and De Maio 2000). The leakage of the dyes was proportional to the concentration of Hsp70 added (Fig. 2b).

Insertion of Hsp70 into liposomes was enhanced by less fluid environments, resulting in oligomerization within the membrane

We also studied the contribution of the fatty acid chain to the incorporation of Hsp70 into the membrane. In this regard, we made liposomes using POPS (one fatty acid chain saturated), dipalmitoylphosphatidylserine (DPPS) (both fatty acid chain saturated) or dioleoylphosphatidylserine (DOPS) (both fatty acid chain unsaturated). The incorporation of Hsp70 into DOPS liposomes was reduced in comparison with POPS liposomes, whereas incorporation into DPPS liposomes was elevated (Fig. 3a). This observation was further corroborated by incubation of liposomes with increasing concentrations of Hsp70. Indeed, Hsp70 incorporation was more robust in the presence of DPPS than POPS (Fig. 3b). This result suggests that the incorporation of Hsp70 into membranes was enhanced by decreasing membrane fluidity; increasing the concentration of Hsp70 that is incorporated into liposomes allowed us to detect high-molecular-weight oligomers by Western blotting even after the addition of LDS and boiling of the sample (Fig. 3b).

The region of Hsp70 inserted into the membrane is toward the C-terminus end

A proteomic approach was used to gain access to the possible domains of Hsp70 that interact with the membrane. Hsp70 contains two major domains: a nucleotide-binding region on the N-terminus end and a substrate-binding site at the C-terminus end, connected by a linker region (Mayer et al. 2000; Chang et al. 2008). Liposomes containing Hsp70 were incubated with chymotrypsin for 2 h at 25 °C. The digested liposomes were sedimented at high-speed centrifugation, and the contents of the vesicles were analyzed by LDS-PAGE and visualized by Coomassie blue staining. Electrophoretic mobility band of approximately 27 kDa were consistently observed after various assays that were not observed when the protein, in absence of liposomes, was digested with the protease. This 27-kDa peptide band was recognized by a polyclonal antibody against Hsp70 as detected by Western blotting (Fig. 4a). The 27-kDa electrophoretic band was excised and analyzed by mass spectroscopy. Between two independent determinations, several peptides overlapping the C-terminus end of Hsp70 were obtained by this procedure (Fig. 4b, indicated by solid or dotted lines). Thus, we assume that the protected proteolytic fragment of Hsp70 is within the C-terminus end of the molecule, which is likely the region that is embedded into the lipid bilayer. This assumption was confirmed by using a chimeric Hsp70 in which glutathione S transferase (GST) was added to the N-terminus of the molecule. Hsp70-GST incorporated well into POPS liposomes, but not into POPC liposomes as the native protein (Fig. 4c). Digestion of liposomes with chymotrypsin resulted in the loss of the GST region, confirming that the N-terminus end is exposed on the external surface of the liposome (Fig. 4d).

Discussion

Although Hsp70 has been detected outside cells in a large number of pathological conditions, the mechanism for the translocation of this protein from the cytosol into the extracellular environment is unclear. We have proposed that the first step in the export mechanism requires the insertion of the protein into the plasma membrane, which is followed by the release associated with vesicles. Since Hsp70 is a large, heavily charged protein, its insertion into membranes is not predicted by thermodynamic approaches. However, several investigations have reported the presence of Hsp70 on the surface of transformed or stressed cells (Multhoff and Hightower 1996; Multhoff 2007; De Maio 2011). Moreover, prior studies have shown that Hsp70 can be inserted into artificial lipid bilayers opening ion conductance pathways (Vega et al. 2008). We developed a simple assay to investigate the process of Hsp70 insertion into membranes by using artificial liposomes. We

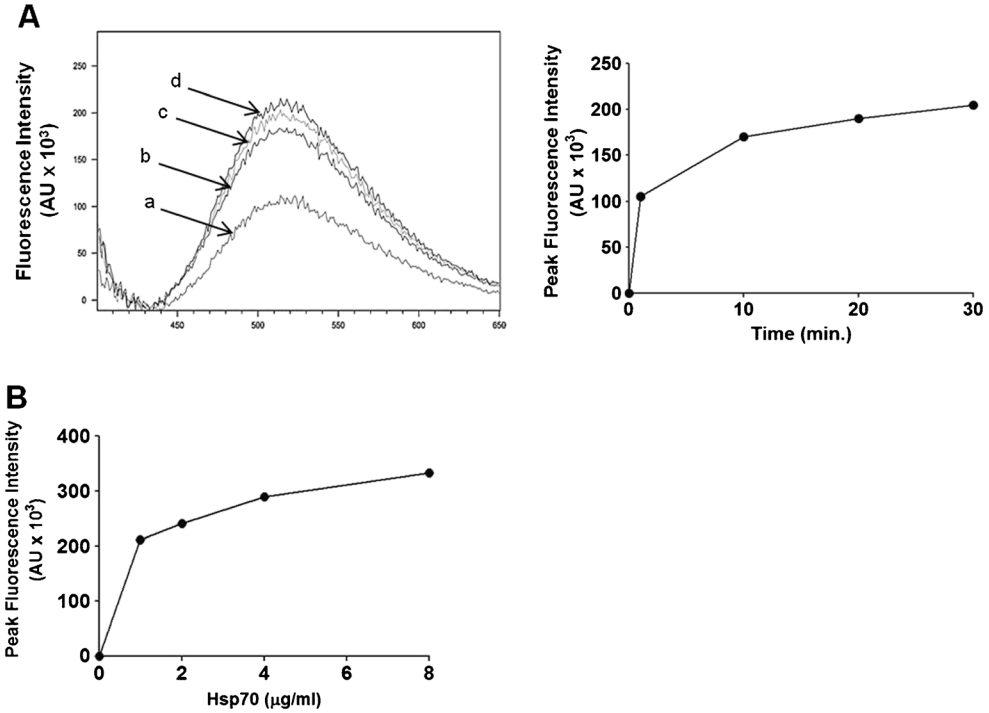

Fig. 2 The incorporation of Hsp70 within liposomes resulted in a disturbance of membrane integrity. Lipids were resuspended in 50-mM Tris–HCl, pH 7.4 containing 50 mM 8-aminonaphthalene-1,3,6-trisulfonate (ANTS) as fluorophore and 50 mM quencher *p*-xylenebis (pyridinium) bromide (DPX) as quencher, extruded through a 200-nm filter 15 times, and passed through a gravity-driven desalting column (Bio-Rad 10-DG) to separate free dye from liposomes. **a** Dye-containing liposomes (POPS/POPC 1:1 ratio) were incubated with Hsp70 at 25 °C for 1 (*a*), 10 (*b*), 20 (*c*), and 30 (*d*) min and the fluorescent spectrum recorded (*left panel*). The maximum peak signal intensity was presented with respect to the time of incubation (*right panel*). **b** Fluorophore-encapsulated liposomes POPS/POPC (1:1) were incubated with increasing concentrations of Hsp70 (1–8 μg/ml) for 30 min at 25 °C. Individual spectra (386-nm excitation) were acquired, and the maximum peak signal intensity was presented with respect to Hsp70 concentrations

found that, indeed, Hsp70 was spontaneously inserted into negatively charged liposomes, but not in liposomes made of neural or positively charged lipids, which is consistent with prior observations regarding Hsp70 lipid specificity (Arispe et al. 2004; Schilling et al. 2009). The incorporation of Hsp70 into the lipid bilayer was enhanced in liposomes with saturated fatty acid lipids, suggesting that the protein is stabilized in less fluid lipid environments. Indeed, Hsp70 has been reported present within detergent resistant microdomains, also known as lipid rafts, in stressed cells (Broquet et al. 2003; Hunter-Lavin et al. 2004; Chen et al. 2005; Vega et al. 2008), which are rich in saturated fatty acids, such as sphingolipids (Simons and Toomre 2000). It is also possible that the presence of saturated fatty acid chains may alter the thickness or the curvature of the membrane, facilitating the insertion of Hsp70. Finally, we observed that the region of Hsp70 inserted into the lipid bilayer is toward the C-terminus end of the molecule, which contains the substrate-binding site. The N-terminus end, which comprises the nucleotide-binding region, was likely exposed to the external region of the liposome, which is consistent with our prior observations indicating that Hsp70/Hsc70 ion channel activity is regulated by the presence of nucleotides (Arispe and De Maio 2000; Vega et al. 2008). Moreover, we have previously shown that Hsp70 in which yellow fluorescent protein (YFP) was added to the C-terminus end of the molecule was not inserted into the plasma membrane (Vega et al. 2008).

A very interesting observation from our studies is that high-molecular-weight oligomers of Hsp70 were observed after incorporation into the lipid bilayer. Indeed, Hsp70/Hsc70 has been reported to form dimers and oligomers (Guidon and Hightower 1986; Benaroudj et al. 1996; Gao et al. 1996; Aprile et al. 2013) in nucleotide- (Kim et al. 1992) and temperature-dependent (Angelidis et al. 1999) forms. The oligomerization region has been proposed as the link between the substrate- and peptide-binding domains (Chang et al. 2008; Aprile et al. 2013). In our working conditions, we observed a predominant monomeric form in solution, whereas oligomers were only detected upon insertion into the lipid bilayer, which is probably due to a conformational change (Wimley et al. 1998), as has been shown for several bacterial proteins (Guilvout et al. 2008; Otzen and Andersen 2013). Thus, Hsp70 oligomerization may be accelerated by compacting the protein within the lipid bilayer, which has been proposed for other β-barrel proteins (Otzen and Andersen 2013) via intermolecular β-strand contacts (Wang et al. 2013). Similarly, antimicrobial peptides are inserted spontaneously into membranes, forming disruptive pores that

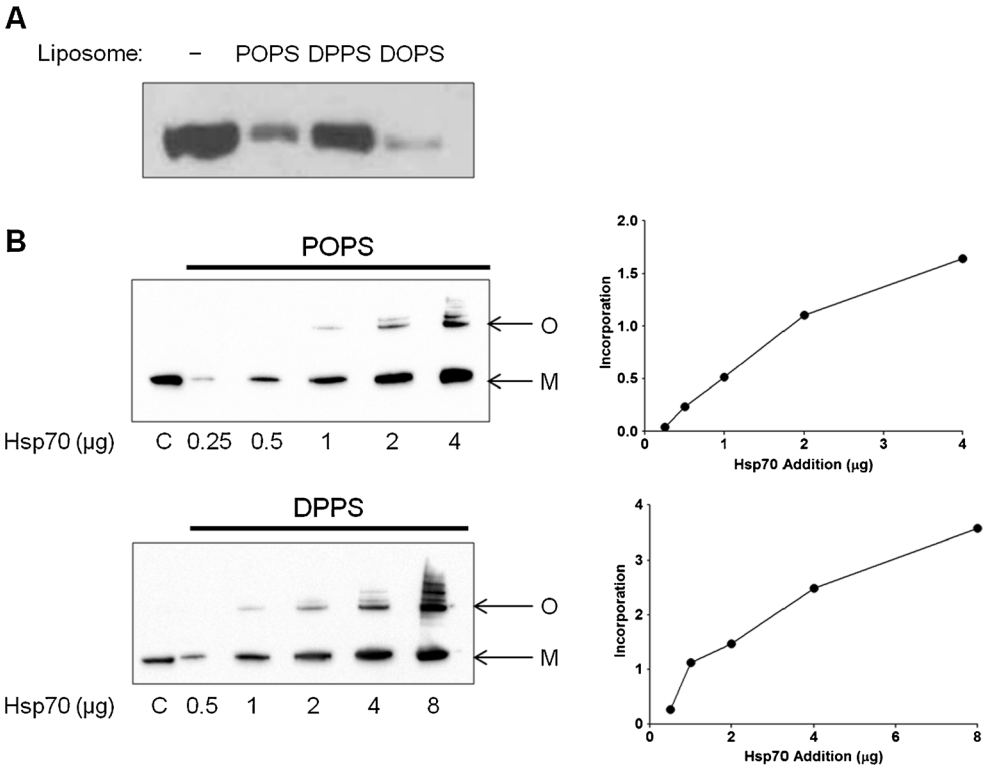

Fig. 3 The incorporation of Hsp70 into liposomes is elevated by increasing the saturation of the fatty acid chain. **a** Pure recombinant Hsp70 (1 μg) was incubated with liposomes made of POPS, DPPS, or DOPS (400 μg) in 50-mM Tris buffer, pH 7.4, for 30 min at 25 °C. At the end of the incubation period, liposomes were centrifuged at 100,000×*g* for 40 min at 4 °C and washed with 100-mM Na_2CO_3, pH 11.5, and centrifuged again. The pellet was resuspended and analyzed by Western blotting a monoclonal antibody against Hsp70. A sample of Hsp70 (1 μg) was included within the Western blot. **b** Various concentrations of Hsp70 (0.25 to 4 μg) were incubated with POPS (400 μg) or Hsp70 (0.5 to 8 μg) with DPPS (400 μg) in 50-mM Tris Buffer, pH 7.4, for 30 min at 25 °C. Proteoliposomes were centrifuged and washed with Na_2CO_3, pH 11.5 as described above and analyzed by Western blotting. The presence of monomers (M) and oligomers (O) is indicated. The signal intensity of each band in the Western blot was quantitated by densitometry using Bio-Rad Image lab Software 4.1. The incorporation into the liposomes was calculated based on the signal of a sample of Hsp70 (1 μg), which was referred to as 1

appear to gain an α helix conformation upon insertion into the lipid bilayer (Bhargava and Feix 2004). Moreover, penetratin, which is also a random-coil monomer in solution, overcame a conformational change from α-helical to β-like conformations in the presence of lipid membranes, resulting in an aggregation that could be visualized in giant unilamellar vesicles (Lee et al. 2010). The prior examples correspond to proteins of low molecular weight. Thus, Hsp70 is an illustration of a large protein that oligomerizes upon insertion into the lipid bilayer.

These observations all together demonstrate that, indeed, Hsp70, despite its highly charged composition, can be inserted into a hydrophobic environment via the C-terminus end of Hsp70, which displayed a β-sheet structure as proposed by x-ray crystallography (Chang et al. 2008). These observations resemble the insertion into membranes by several bacterial proteins. These proteins, known as outer membrane proteins, are also inserted into the membrane by a β-barrel domain (Schulz 2000; Otzen and Andersen 2013). A few mammalian proteins, which also display β-sheet structure, have been reported to be inserted into lipid bilayers, including annexin (Rojas et al. 1992), amylin (Mirzabekov et al. 1996), the human voltage-dependent anion-selective channel (Shanmugavadivu et al. 2007), and the amyloid β peptide (Arispe et al. 1993, 1996). Some of these β-barrel proteins form oligomeric complexes that are resistant to SDS solubilization (Stanley and Fleming 2008), which resemble our observation with Hsp70 within the lipid membrane. The spontaneous incorporation of polypeptides into membranes has been postulated as involving sequential steps, including the interaction of the peptide with the lipid head and change in peptide conformation that facilitates the insertion into the membrane, which is followed by oligomerization in some cases (Wimley et al. 1998). For example, cholesterol-dependent cytolysins' insertion into membranes is initiated by the initial recognition of cholesterol moieties, which is followed by insertion, lateral diffusion, and formation of amphipathic β-sheets, which are the product of a major conformational change from α-helices into β-strands (Shatursky et al. 1999; Shepard et al. 2000) in an orderly manner

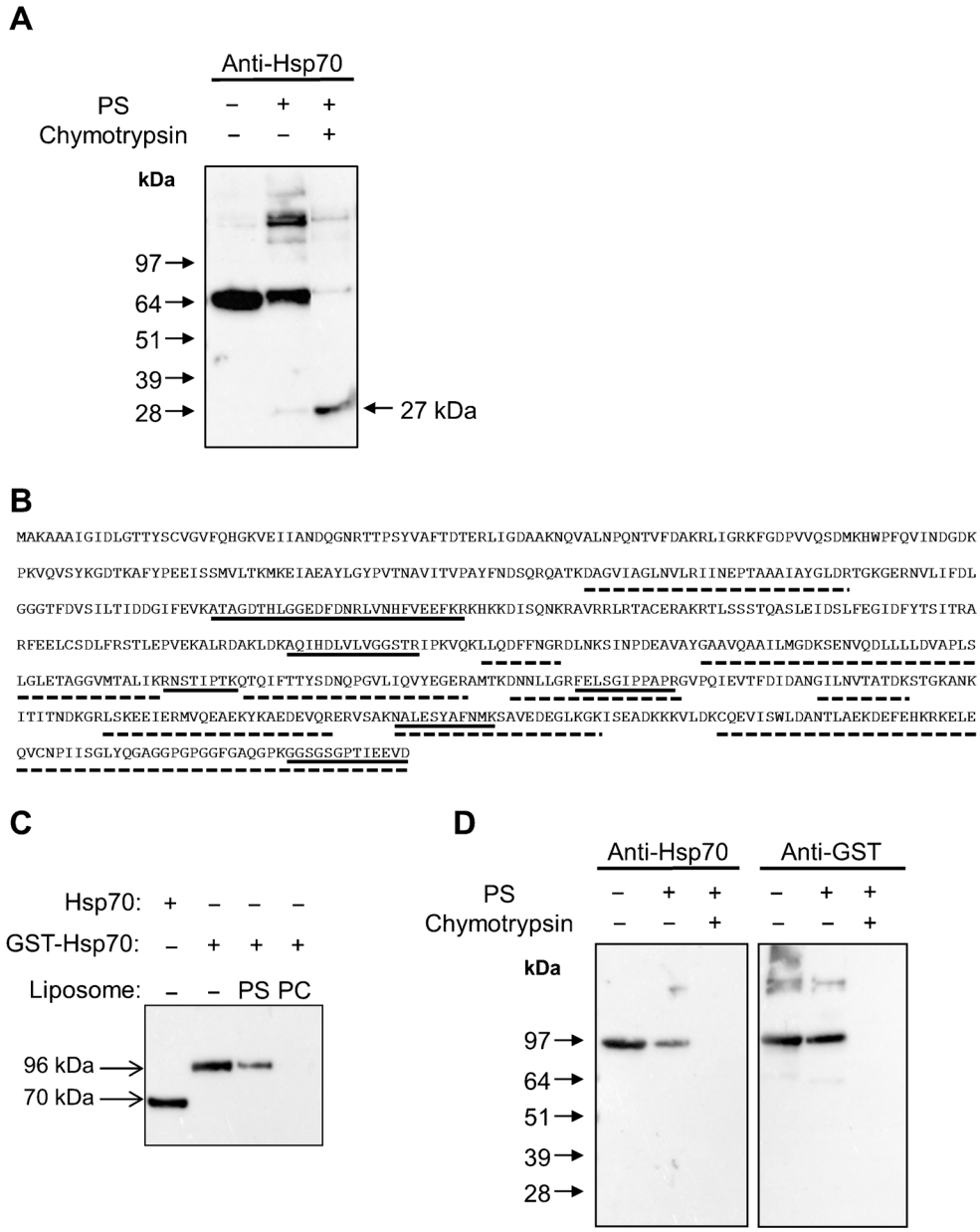

Fig. 4 Hsp70 is inserted into liposomes via the C-terminus end of the protein. Recombinant Hsp70 was incorporated into POPS liposomes in 50 mM Tris buffer, pH 7.4 for 30 min at 25 °C. At the end of the incubation period, liposomes were centrifuged at 100,000×*g* for 40 min at 4 °C and washed with 100-mM Na_2CO_3, pH 11.5. The proteoliposomes were digested or not with chymotrypsin (2 mM) for 2 h at 25 °C and centrifuged again at 100,000×*g* for 40 min at 4 °C. Proteins that were retained within liposomes were separated by LDS-PAGE and stained with Coomassie blue. A band of apparent electrophoretic mobility corresponding to a proteolytic fragment of 27 kDa was detected in the chymotrypsin-digested sample, but not in chymotrypsin-digested Hsp70. **a** The 27-kDa band was detected by a polyclonal antibody against Hsp70 (SPA812, assay designs) as visualized by Western blotting. **b** The 27-kDa proteolytic fragment was excised from the gel, digested with trypsin, and resulting peptides were analyzed by HPLC coupled with tandem mass spectrometry (LC-MS/MS) using nano-spray ionization (TripleTOF 5600 hybrid mass spectrometer (AB SCIEX)). Data were analyzed using MASCOT® (Matrix Science) and Protein Pilot 4.0 (AB SCIEX) for peptide identifications; *solid and dashed lines* correspond to the mapped peptides from two independent determinations. **c** GST-tagged Hsp70 at the N-terminus end of the protein was incubated with POPS or POPC liposomes as described above and analyzed by Western blotting using anti-Hsp70 monoclonal antibody (SPA810, Assay Designs). Recombinant Hsp70 was used as a positive control. **d** GST-tagged Hsp70 at the N-terminus end of the protein was incorporated into POPS liposomes and treated or not with chymotrypsin for 2 h at 25 °C. The samples were centrifuged as described above. The digested liposome samples were analyzed by Western blotting using anti-Hsp70 monoclonal antibody (SPA810, Assay Designs) or anti GST antibody (Clone Dg122-2A7, Millipore)

(Shatursky et al. 1999; Shepard et al. 2000; Heuck et al. 2000). It could be argued that a highly charged sequence of Hsp70 contradicts any possibility of insertion into a lipid bilayer due to the great energetic cost of introducing a charged amino acid

within a hydrophobic environment (Wimley et al. 1998; Schow et al. 2011). However, arginines within the S4 helix of the voltage-gated ion channel are exposed to lipids within the membrane as assumed from the crystallographic structure (Jiang et al. 2003; Lee et al. 2005; Long et al. 2005, 2007) as well as by other methodologies (Witte et al. 2013). The insertion of Hsp70 into lipid membranes could be explained by the assumption that the lipid bilayer is heterogeneous, containing a very hydrophobic core in the center of the membrane surrounded by a more heterogeneous environment containing polar lipid heads and water (Wiener and White 1992; White and Wimley 1998). This heterogeneous region could likely house the charged amino acids. Therefore, it is possible that Hsp70 is initially associated with this more polar region of the lipid bilayer, which could be enhanced by the interaction of the negatively charged head groups of PS with several positive amino acid regions of Hsp70. It is also possible that the translocation of Hsp70 into the lipid bilayer may be related to the flipping/flopping of PS across the membrane (De Maio 2011). In this regard, amyloid β peptides have been shown to alter the mobility of PS during interaction with membranes (Buchsteiner et al. 2010).

Based on the preceding observations, we propose that Hsp70, which is located in the cytosol, recognizes PS moieties on the inner side of the plasma membrane, which is followed by a change of conformation and insertion into the lipid bilayer. Upon membrane insertion, Hsp70 oligomerizes and moves into lipid rafts, exposing a small region of the C-terminus end to the extracellular environment. Indeed, the presence of Hsp70 on the surface of cells could only be accomplished by using antibodies against the C-terminus end (Botzler et al. 1998). If this prediction is correct, the N-terminus end of Hsp70, which contains the ATP-binding site, should be exposed to the cytosolic side of the plasma membrane. This membrane topology is consistent with early observations indicating that the channel activity of Hsc70 and Hsp70 was regulated by nucleotides, suggesting that this domain should be exposed to the aqueous environment (Arispe and De Maio 2000; Vega et al. 2008). Additionally, the present study is also consistent with the exposure of the N-terminus end on the external part of the liposome. Recently, another study proposed that Hsp70 interacts peripherally with lipid membranes (Mahalka et al. 2014), which is different from our findings. It could be that Hsp70 has different forms of lipid interactions. Perhaps Mahalka et al. (2014) were detecting the initial interaction of the monomeric form of Hsp70 with phospholipids. In our working conditions, several approaches were used to demonstrate that Hsp70 is, indeed, embedded into the lipid bilayer, including washes at high or low pH, which remove any associated protein to the membrane surface. The resistance of Hsp70 within membranes to acid or basic washes is consistent with prior observations describing the presence of this protein on the plasma membrane of transformed cells (Multhoff et al. 1997, Gastpar et al. 2005, Vega et al. 2008).

We have postulated that the insertion of Hsp70 into the plasma membrane is the gateway for its export in the form of vesicles. Certainly, several investigators have reported the presence of Hsp70 within ECV (De Maio 2011; De Maio and Vazquez 2013). ECV could be derived by various mechanisms, including endocytosis (exosomes) or ectocytosis (ectosomes). They contain membrane components and cargo, which could deliver these molecules to other cells by membrane fusion or endocytosis (Thery et al. 2009; De Maio 2011). Indeed, ECV are likely part of an alternative mechanism for cellular communication in which signals from one group of cells are released into the extracellular environment, delivering information regarding the metabolic condition of the organism to other target cells, perhaps to maintain homeostasis and proteostasis. During stress conditions such as injury or infection, these vesicles may deliver stress signals, such as Hsp70, to alert the organism that an insult has occurred, activating the necessary machinery to confront and avoid the propagation of the harmful conditions. We have coined this mechanism the "Stress Observation System" (De Maio 2011). Thus, the translocation of Hsp70 into the plasma membrane and its release associated with vesicles could be part of this systemic mechanism of stress surveillance. The mechanism of membrane insertion prior to the release into the extracellular milieu could explain the export of many other proteins that lack a secretory peptide signal.

In summary, our observations provide evidence of an unexpected ability of a large, charged protein to become inserted into a lipid membrane. This observation provides a new paradigm for the interaction of proteins with lipid environments. Moreover, it opens the possibility for further investigations regarding the mechanism of protein lipid interaction and insertion that were limited in the past due to the use of small proteins or peptides.

Acknowledgments This work was supported by the National Institutes of Health, grant numbers GM R01 098455 and GM R25 083275. We would like to thank Dr. Majid Ghassemian for the mass spectrometry analysis. We are also grateful to Dr. C.C. King for providing us with Hsp70-GST construct. The editorial assistance of Molly Wofford is also appreciated.

References

Angelidis CE, Lazaridis I, Pagoulatos GN (1999) Aggregation of hsp70 and hsc70 in vivo is distinct and temperature-dependent and their chaperone function is directly related to non-aggregated forms. Eur J Biochem 259:505–512

Aprile FA, Dhulesia A, Stengel F, Roodveldt C, Benesch JL, Tortora P, Robinson CV, Salvatella X, Dobson CM, Cremades N (2013) Hsp70 oligomerization is mediated by an interaction between the

interdomain linker and the substrate-binding domain. PLoS One 8: e67961
Arispe N, De Maio A (2000) ATP and ADP modulate a cation channel formed by Hsc70 in acidic phospholipid membranes. J Biol Chem 275:30839–30843
Arispe N, Pollard HB, Rojas E (1993) Giant multilevel cation channels formed by Alzheimer disease amyloid beta-protein [A beta P-(1–40)] in bilayer membranes. Proc Natl Acad Sci U S A 90:10573–10577
Arispe N, Pollard HB, Rojas E (1996) Zn2+ interaction with Alzheimer amyloid beta protein calcium channels. Proc Natl Acad Sci U S A 93:1710–1715
Arispe N, Doh M, Simakova O, Kurganov B, De Maio A (2004) Hsc70 and Hsp70 interact with phosphatidylserine on the surface of PC12 cells resulting in a decrease of viability. FASEB J 18:1636–1645
Benaroudj N, Triniolles F, Ladjimi MM (1996) Effect of nucleotides, peptides, and unfolded proteins on the self-association of the molecular chaperone HSC70. J Biol Chem 271:18471–18476
Bhargava K, Feix JB (2004) Membrane binding, structure, and localization of cecropin-mellitin hybrid peptides: a site-directed spin-labeling study. Biophys J 86:329–336
Botzler C, Schmidt J, Luz A, Jennen L, Issels R, Multhoff G (1998) Differential Hsp70 plasma-membrane expression on primary human tumors and metastases in mice with severe combined immunodeficiency. Int J Cancer 77:942–948
Broquet AH, Thomas G, Masliah J, Trugnan G, Bachelet M (2003) Expression of the molecular chaperone Hsp70 in detergent-resistant microdomains correlates with its membrane delivery and release. J Biol Chem 278:21601–21606
Buchsteiner A, Hauss T, Dante S, Dencher NA (2010) Alzheimer's disease amyloid-beta peptide analogue alters the ps-dynamics of phospholipid membranes. Biochim Biophys Acta 1798: 1969–1976
Chang YW, Sun YJ, Wang C, Hsiao CD (2008) Crystal structures of the 70-kDa heat shock proteins in domain disjoining conformation. J Biol Chem 283:15502–15511
Chen S, Bawa D, Besshoh S, Gurd JW, Brown IR (2005) Association of heat shock proteins and neuronal membrane components with lipid rafts from the rat brain. J Neurosci Res 81:522–529
De Maio A (1999) Heat shock proteins: facts, thoughts, and dreams. Shock 11:1–12
De Maio A (2011) Extracellular heat shock proteins, cellular export vesicles and the Stress Observation System: a form of communication during injury, infection, and cell damage. Cell Stress Chaperones 16:235–249
De Maio A, Vazquez D (2013) Extracellular heat shock proteins: a new location, a new function. Shock 40:239–246
Gable JE, Schlamadinger DE, Cogen AL, Gallo RL, Kim JE (2009) Fluorescence and UV resonance raman study of peptide-vesicle interactions of human cathelicidin LL-37 and its F6W and F17W mutants. Biochemistry 48:11264–11272
Gao B, Eisenberg E, Greene L (1996) Effect of constitutive 70-kDa heat shock protein polymerization on its interaction with protein substrate. J Biol Chem 271:16792–16797
Gastpar R, Gehrmann M, Bausero MA, Asea A, Gross C, Schroeder JA, Multhoff G (2005) Heat shock protein 70 surface-positive tumor exosomes stimulate migratory and cytolytic activity of natural killer cells. Cancer Res 65:5238–5247
Guidon PT Jr, Hightower LE (1986) Purification and initial characterization of the 71-kilodalton rat heat-shock protein and its cognate as fatty acid binding proteins. Biochemistry 25:3231–3239
Guilvout I, Chami M, Berrier C, Ghazi A, Engel A, Pugsley AP, Bayan N (2008) In vitro multimerization and membrane insertion of bacterial outer membrane secretin PulD. J Mol Biol 382:13–23
Hartl FU, Hayer-Hartl M (2009) Converging concepts of protein folding in vitro and in vivo. Nat Structural Mol Biol 16:574–581
Heuck AP, Hotze EM, Tweten RK, Johnson AE (2000) Mechanism of membrane insertion of a multimeric beta-barrel protein: perfringolysin O creates a pore using ordered and coupled conformational changes. Mol Cell 6:1233–1242
Hightower LE, Guidon PT Jr (1989) Selective release from cultured mammalian cells of heat-shock (stress) proteins that resemble glia-axon transfer proteins. J Cell Physiol 138:257–266
Hunter-Lavin C, Davies EL, Bacelar MM, Marshall MJ, Andrew SM, Williams JH (2004) Hsp70 release from peripheral blood mononuclear cells. Biochem Biophys Res Commun 324:511–517
Jiang Y, Lee A, Chen J, Ruta V, Cadene M, Chait BT, MacKinnon R (2003) X-ray structure of a voltage-dependent K+channel. Nature 423:33–41
Kim D, Lee YJ, Corry PM (1992) Constitutive HSP70: oligomerization and its dependence on ATP binding. J Cell Physiol 153:353–361
Lee SY, Lee A, Chen J, MacKinnon R (2005) Structure of the KvAP voltage-dependent K+channel and its dependence on the lipid membrane. Proc Natl Acad Sci U S A 102:15441–15446
Lee CC, Sun Y, Huang HW (2010) Membrane-mediated peptide conformation change from alpha-monomers to beta-aggregates. Biophys J 98:2236–2245
Long SB, Campbell EB, MacKinnon R (2005) Crystal structure of a mammalian voltage-dependent Shaker family K+channel. Science 309:897–903
Long SB, Tao X, Campbell EB, MacKinnon R (2007) Atomic structure of a voltage-dependent K+channel in a lipid membrane-like environment. Nature 450:376–382
Mahalka AK, Kirkegaard T, Jukola LT, Jaatela M, Kinnunen PKJ (2014) Human heat shock protein 70 (Hsp70) as a peripheral membrane protein. Biochim Biophys Acta 1838:1344–1361
Mayer MP, Schroder H, Rudiger S, Paal K, Laufen T, Bukau B (2000) Multistep mechanism of substrate binding determines chaperone activity of Hsp70. Nat Struct Biol 7:586–593
Mirzabekov TA, Lin MC, Kagan BL (1996) Pore formation by the cytotoxic islet amyloid peptide amylin. J Biol Chem 271:1988–1992
Multhoff G (2007) Heat shock protein 70 (Hsp70): membrane location, export and immunological relevance. Methods 43:229–237
Multhoff G, Hightower LE (1996) Cell surface expression of heat shock proteins and the immune response. Cell Stress Chaperones 1:167–176
Multhoff G, Botzler C, Jennen L, Schmidt J, Elwart J, Issels R (1997) Heat shock protein 72 on tumor cells: a recognition structure for natural killer cells. J Immunol 158:4341–4350
Nickel W, Seedorf M (2008) Unconventional mechanisms of protein transport to the cell surface of eukaryotic cells. Annu Rev Cell Dev Biol 24:287–308
Otzen DE, Andersen KK (2013) Folding of outer membrane proteins. Arch Biochem Biophys 531:34–43
Rojas E, Arispe N, Haigler HT, Burns AL, Pollard HB (1992) Identification of annexins as calcium channels in biological membranes. Bone Miner 17:214–218
Schilling D, Gehrmann M, Steinem C, De Maio A, Pockley AG, Abend M, Molls M, Multhoff G (2009) Binding of heat shock protein 70 to extracellular phosphatidylserine promotes killing of normoxic and hypoxic tumor cells. FASEB J 23:2467–2477
Schow EV, Freites JA, Cheng P, Bernsel A, von Heijne G, White SH, Tobias DJ (2011) Arginine in membranes: the connection between molecular dynamics simulations and translocon-mediated insertion experiments. J Membr Biol 239:35–48
Schulz GE (2000) Beta-Barrel membrane proteins. Curr Opin Struct Biol 10:443–447
Shanmugavadivu B, Apell HJ, Meins T, Zeth K, Kleinschmidt JH (2007) Correct folding of the beta-barrel of the human membrane protein VDAC requires a lipid bilayer. J Mol Biol 368:66–78
Shatursky O, Heuck AP, Shepard LA, Rossjohn J, Parker MW, Johnson AE, Tweten RK (1999) The mechanism of membrane insertion for a

cholesterol-dependent cytolysin: a novel paradigm for pore-forming toxins. Cell 99:293–299

Shepard LA, Shatursky O, Johnson AE, Tweten RK (2000) The mechanism of pore assembly for a cholesterol-dependent cytolysin: formation of a large prepore complex precedes the insertion of the transmembrane beta-hairpins. Biochemistry 39:10284–10293

Simons K, Toomre D (2000) Lipid rafts and signal transduction. Nat Rev Mol Cell Biol 1:31–39

Stanley AM, Fleming KG (2008) The process of folding proteins into membranes: challenges and progress. Arch Biochem Biophys 469:46–66

Thery C, Ostrowski M, Segura E (2009) Membrane vesicles as conveyors of immune responses. Nat Rev Immunol 9:581–593

Vega VL, Rodriguez-Silva M, Frey T, Gehrmann M, Diaz JC, Multhoff G, Arispe N, De Maio A (2008) Hsp70 translocates into the plasma membrane after stress and is released into the extracellular environment in a membrane-associated form that activates macrophages. J Immunol 180:4299–4307

Wang H, Andersen KK, Vad BS, Otzen DE (2013) OmpA can form folded and unfolded oligomers. Biochim Biophys Acta 1834:127–136

White SH, Wimley WC (1998) Hydrophobic interactions of peptides with membrane interfaces. Biochim Biophys Acta 376:339–352

Wiener MC, White SH (1992) Structure of a fluid dioleoylphosphatidylcholine bilayer determined by joint refinement of x-ray and neutron diffraction data. III. Complete structure. Biophys J 61:434–447

Wimley WC, Hristova K, Ladokhin AS, Silvestro L, Axelsen PH, White SH (1998) Folding of beta-sheet membrane proteins: a hydrophobic hexapeptide model. J Mol Biol 277:1091–1110

Witte K, Olausson BE, Walrant A, Alves ID, Vogel A (2013) Structure and dynamics of the two amphipathic arginine-rich peptides RW9 and RL9 in a lipid environment investigated by solid-state NMR and MD simulations. Biochim Biophys Acta 1828:824–833