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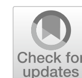
MINI REVIEW



The interaction of heat shock proteins with cellular membranes: a historical perspective

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Abstract
The interaction of heat shock proteins (HSP) with cellular membranes has been an enigmatic process, initially observed by morphological studies, inferred during the purification of HSP70s, and confirmed after the detection of these proteins on the surface of cancer cells and their insertion into artificial lipid bilayers. Today, the association of several HSP with lipid membranes is well established. However, the mechanisms for membrane insertion have been elusive. There is conclusive evidence indicating that HSP70s have a great selectivity for negatively charged phospholipids, whereas other HSP have a broader spectrum of lipid specificity. HSP70 also oligomerizes upon membrane insertion, forming ion conductance channels. The functional role of HSP70 lipid interactions appears related to membrane stabilization that may play a role during cell membrane biogenesis. They could also play a role as membrane chaperones as well as during endocytosis, microautophagy, and signal transduction. Moreover, HSP membrane association is a key component in the extracellular export of these proteins. The presence of HSP70 on the surface of cancer cells and its interaction with lysosome membranes have been envisioned as potential therapeutic targets. Thus, the biology and function of HSP membrane association are reaching a new level of excitement. This review is an attempt to preserve the recollection of the pioneering contributions of many investigators that have participated in this endeavor.

Keywords Heat shock proteins · HSP70 · HSPA · Membranes · Phospholipids · Cellular stress

The heat shock response: a tale of rejection

Science, like many other disciplines, is operated with unwritten rules, some of them transmitted from generation to generation, and others shaped by rejection, flout, and recognition. The most important tenet is that scientific claims need to be supported by solid evidence. In some circumstances, new findings contradict conventional wisdom, and they are rejected or ignored. This aspect was clearly noticed in R. J. Ellis's words "It is my belief that scientists should resist the natural tendency to ignore unexpected observations that do not fit the existing paradigm, but take the risk of pursuing them in hope that they lead to new ideas and discoveries" (Ellis 1996). Certainly, these circumstances have impacted and shaped the progress of the stress response and heat shock protein biology. The story began in the early 1960s, when a talented Italian investigator, Ferruccio Ritossa, found that *Drosophila* cells exposed to elevated temperatures responded with a robust chromosomal activity, which was confirmed by subsequent experiments. This observation was rejected because it was labeled as "irrelevant to the scientific community" (Ritossa 1962, 1996; De Maio et al. 2012). Why did Ritossa's manuscript receive this indifferent response from a high-impact journal? We may never know the details as Ritossa did not elaborate in print on the original review prior to his passing in 2014. However, we can reflect upon the times. The biological models that dominated

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molecular genetics were *E. coli* and its phage lambda. The early 1960s was part of the golden age of molecular biology, often stylized by the quotation frequently ascribed to Jacques Monod that “What is true for *E. coli* is true for the elephant.” The famous PaJaMa experiments had been published in 1959 by Arthur Pardee, Francois Jacob, and Jacques Monod. The PaJaMa experiments strengthened the hypothesis that a specific molecule facilitated the production of proteins from DNA. This was followed in 1961 by Jacob and Monod’s paper titled “Genetic Regulatory Mechanisms of the Synthesis of Proteins,” showing how genes could be activated to make a specific enzyme β-galactosidase. Gene expression seemed so precise and selective with specific inducer molecules inactivating specific repressors on specific genes, and it appeared to extend throughout many if not all species. How could thermal energy, capable of being absorbed by any molecule and therefore the antithesis of specificity, initiate these remarkable processes to activate a specific set of genes in *Drosophila* cells and ultimately found in eukaryotic and prokaryotic cells in general? This simple question became a stumbling block even for investigators who accepted the premise that thermal energy increases in cells could induce the expression of a specific set of proteins.

Ritossa’s initial finding was forgotten for almost 12 years, and when it was recalled, colleagues spoke of it at best as a curiosity of *Drosophila* biology and at worst as a laboratory artifact. Then the proteins that were expressed in response to high temperatures were identified by Alfred Tissieres and collaborators (Tissieres et al. 1974). Alfred, during a sabbatical leave with Hershel Mitchell, had not intended to search for the heat shock proteins (HSP), as they became known, but his original project had not worked, and he was running out of time to test a new polyacrylamide gel method, so he decided to do a quick experiment to find them. It is quite possible that the *Drosophila* heat shock genes would not have been selected as models of eukaryotic gene expression had not been known due to this happenstance that those genes actually encoded proteins.

Clues to the functions of heat shock proteins

A few years after the discovery of the proteins, during the bloom of molecular biology, the genes encoding HSP were cloned (Schedl et al. 1978; Livak et al. 1978; Craig et al. 1979), and the mechanisms of transcription regulation were elucidated (Pelham 1982; Wu 1984; Bahl et al. 1987). There was little interest in attempting to discover the functions of the HSP, and in fact, there were no solid clues to what they might be doing in cells. The fact that virtually all molecules absorb thermal energy and are affected by it, even if only to increase the kinetic energy, meant that no clues were provided by the major known inducer. Promising new clues came from two unlikely fields, animal virology, and neuroscience. Lawrence Hightower, while studying Newcastle Disease Virus-infected avian cell cultures, serendipitously found that different amino acid analogs sharing the common property of incorporating into aberrant proteins altering functions and stabilities caused the induction of HSP at normal temperatures. Independently, Fredric White, while studying rat brain slices as in vitro models for protein synthesis, discovered a small set of proteins, rapidly induced in this tissue, that he ultimately determined to be the mammalian equivalent of the *Drosophila* HSP. He suggested that these proteins were induced in response to the trauma of tissue slicing and incubation in vitro. They jointly published their observations showing that amino acid analogs and tissue trauma induced the same set of proteins in mammalian cells (Hightower and White 1981). A great step that followed was the discovery that the expression of HSP was not limited to lower organisms, tissue preparations, and cells in culture, but also found in mammalian tissues after in vivo hyperthermia (Currie and White 1983). Suddenly, it became possible to test hypotheses that cells were capable of “sensing” the presence of damaged or unfolded proteins and responding by producing cellular defense proteins to meet the challenge. Essentially any stressor capable of causing cellular or tissue damage that directly or indirectly caused the accumulation of abnormal proteins could be an inducer of the heat shock response (Hightower 1980; Ananthan et al. 1986; Edington et al. 1989). Then, HSP were recognized as composed of many different polypeptides with different molecular masses, some of which were constitutively present under normal physiological conditions, whereas others were induced after a variety of stressors (Lindquist 1986; Lindquist and Craig 1988). Then, the new concept of proteotoxic stress was born (Hightower 1991). A subsequent major breakthrough was related to the finding that HSP participate in protein folding during normal physiological conditions as well as after harmful events, and the concept of molecular chaperones was introduced in this context (Ellis 1996), resembling a prior concept coined by Laskey et al. (1978) regarding a nuclear protein, nucleoplasmin, preventing the aggregation of histones during nucleosome assembly. The folding capacity of HSP was related to an intrinsic ATPase activity. For example, Sadis and Hightower (1992) used the unfolded precursor protein apocytochrome c to show that HSP70 and its constitutively expressed cognate HSC70 can distinguish between unfolded and folded forms of the protein. In this case, the HSP70/HSC70 ATPase activity was only stimulated by the unfolded form. Moreover, the old notion of hyperthermia tolerance observed during approaches to eradicate malignant tumors, initially reported by Crile (1963), was indeed mediated by HSP, a process coined “stress tolerance” (Landry et al. 1982; Subjeck et al. 1982; Li and Werb 1982), which gave a new

perspective to the field. During the following years, a great deal of effort was directed at purifying the proteins (Welch and Feramisco 1982; Guidon and Hightower 1986a, b) and developing specific antibodies (Welch and Feramisco 1984; Welch and Suhan 1985; 1986). With these tools on hand, the biology of HSP flourished, resulting in a very exciting period of discovery that continues to the present and it is impulse into the future.

The encounter of heat shock proteins with membranes

Morphological studies for the detection of HSP within cells revealed the presence of these proteins in various subcellular compartments, including in close proximity to membranes (Velazquez et al. 1980; Velazquez and Lindquist 1984). The apparent presence of HSP within membranes was also later observed by others (LaThangue 1984; Welch and Suhan 1985). Although the potential interaction of the proteins with membranes was not further investigated, a surprising observation was encountered during the purification of rat HSP70/HSC70 from cellular extracts. Guidon and Hightower (1986a; b) found that the purified protein was still associated with fatty acids. This observation became the first solid evidence for the interaction of HSP70s with lipids. These pioneering observations were also forgotten for many years, and the attention was directed at the role of HSP70 in protein folding and thermotolerance. It was relatively easy for skeptics to dismiss the association of noncovalently associated fatty acids with HSP as simply a gratuitous presence of a small amount of unesterified and nonspecific fatty acids in purified protein preparations. This was despite the fact that the same fatty acids, palmitic and stearic acids in the same 1:1 ratio, were associated with purified HSC70 and HSP70 from two organs, liver and brain, with very different free fatty acid compositions (Guidon and Hightower 1986a; b). The interest in the association of HSP with membranes was regained by observations regarding the presence of these proteins on the cell surface. The first report on this occurrence was in 1992, in which HSP90 and HSP70 were detected on the surface of several tumor cell lines (Ferrarini et al. 1992). Additionally, HSP70 was detected on the surface of retroocular fibroblasts obtained from patients suffering from Graves' ophthalmopathy, an autoimmune inflammatory disorder (Heufelder et al. 1992). Moreover, HSP70 was also found in T cell lines infected with leukemia virus I, triggering the production of antibodies against the HSP (Chouchane et al. 1994). These early observations did not receive any major attention, probably because it was unknown whether the protein was inserted into the membrane or just associated with plasma membrane proteins. It was not until Gabriele Multhoff 's remarkable work showing in very elegant studies that HSP70 was exclusively present on the surface of tumor cells, embedded into the plasma membrane (Multhoff et al. 1995). This annotation was very controversial at that time, particularly because the majority of available antibodies did not recognize the protein on the cell surface, except for one commercially available, which was rapidly discontinued, probably due to the lack of business. Multhoff 's group performed an epitope mapping of HSP70, identifying a motif coined "TKD" (TKDNNLLGRFELSG) that was exposed outside the cell (Botzler et al. 1998). A new antibody for this epitope was raised and distributed, allowing several groups to confirm Multhoff's initial findings. Today, there are extensive reports demonstrating the presence of several HSP on the surface of various cells (Table 1). Moreover, there are several excellent reviews on the topic (Multhoff and Hightower 2011; De Maio 2011; De Maio and Vazquez 2013; Shevtsov et al. 2020; Elmallah et al. 2020).

The controversial finding that HSP70 was inserted into the plasma membrane of cancer cells was again unappreciated for many years. The turning point came in the year 2000 at the annual Cold Spring Harbor Meeting "Molecular chaperones and the heat shock response," in which two posters changed the course of the field. Asea and Calderwood showed elegant studies demonstrating that exogenous HSP70 was capable of activating macrophages producing a robust inflammatory response. This study was later published in a prestigious journal (Asea et al. 2000). This observation opened an extensive line of investigation regarding the role of extracellular HSP in cell signaling and as biomarkers that is still very active today (Calderwood et al. 2007a; De Maio 2011, 2014; Pockley et al. 2014). The second poster by Arispe and De Maio showed that HSC70 (HSPA8) could get inserted into planar lipid bilayers, forming a very stable ion channel with a conductance regulated by nucleotides. The poster was greeted by a very seasoned investigator who shouted at one of the presenters during the initial lunch, "Are you saying that HSP70 is opening pores? Are you crazy?" This observation was later published in the Journal of Biological Chemistry after being rejected by a prominent journal because it did not have any biological importance (Arispe and De Maio 2000). The Arispe and De Maio poster did not cause any major impact at that time, perhaps because there was no other electrophysiologist at the meeting. However, two people were very excited about the observation. The first one was Michael Tytell, who, many years back, showed that a heat shock-like protein was released from the squid giant axon and transferred to the glia (Tytell et al. 1986). The second was Larry Hightower, who previously showed, as indicated above, that the protein was associated with fatty acids (Guidon and Hightower 1986a, b). Thus, the association of HSC70 with membranes could nicely explain their original findings.

Table 1 The presence of several HSP on the surface of various cells

New name	Alternative name	References
HSPA1	HSP70	Ferrarini et al. (1992); Heufelder et al. (1992); Chouchane et al. (1994); Multhoff et al. (1995); Takashima et al. (1996); Botzler et al. (1998); Kaur et al. (1998); Camins et al. (1999); Hantschel et al. (2000); Farkas et al. (2003); Bausero et al. (2004); Gehrmann et al. (2008); Vega et al. (2008); Sedlackova et al. (2009); Tani et al. (2009); Lasunskaia et al. (2010); Bilog et al. (2019)
HSPA5	Grp78, BIP	Takemoto et al. (1992); Berger et al. (1997); Delpino and Castelli (2002); Arap et al. (2004); Zhang et al. (2010; 2013); Kang et al. (2016); Toyoda et al. (2018); Naaby-Hansen and Herr (2010)
HSPA6	HSP70B	Noonan et al. (2008)
HSPA8	Hsc70	Mills et al. (2010)
HSPC	Hsp90	Ferrarini et al. (1992); Camins et al. (1999); Kakimura et al. (2002); Tsutsumi and Neckers (2007); Fong-ngern et al. (2016); Lauwers et al. (2018)
HSPC4	GRP94/ Grp96	Altmeyer et al. (1996); Robert et al. (1999); Toyoda et al. (2018)
HSPC3	Hsp90beta	Cid et al. (2004; 2005; 2009); Sidera et al. (2004); Gronthos et al. (2009)
HSPC2	Hsp90alpha	Sidera et al. (2004)
HSPD	HSP60	Torok et al. (1997); Belles et al. (1999); Naaby-Hansen and Herr (2010)
HSPB	Hsp17	Laskowska et al. (2004); Tsvetkova et al. (2002)
HSPB1	Hsp27	Camins et al. (1999); Bausero et al. (2004)
HSPB5	alpha-crystallin	Tjondro et al. (2016); Borchman and Tang (1996); Ifeanyi and Takemoto (1991); Tsvetkova et al. (2002)

The interaction of HSP70s with lipids and membranes

Following the pioneering work of Guidon and Hightower, two publications appeared. Alder et al. (1990) reported that the addition of HSP70 to liposomes produced a leakage of the vesicle contents, probably due to the formation of pores. Moreover, Negulyaev et al. 1996 found that the addition of exogenous HSP70 to patch-clamped membranes activated potassium currents. These observations were under the radar for many years. It was not until 2000 that Arispe and De Maio observed that HSC70 (HSPA8) could form very stable and uniform ion conductance channels upon incorporation into artificial lipid bilayers. The ion conductance pathway displayed a multi-conductance activity by frequently switching between different open levels. The channel was selective for cations, and it was not voltage-dependent. Moreover, the channel conductivity was opened by ATP and closed by ADP (Arispe and De Maio 2000). The HSPA8 channel activity was later confirmed by Macazo and White (2014), and a similar channel activity was also reported for HSPA1 (Vega et al. 2008).

An interesting feature of the interaction of HSP70 with lipid membranes was their high selectivity for negatively charged phospholipids, particularly phosphatidylserine (PS) (Arispe et al. 2004; Schilling et al. 2009; Armijo et al. 2014; Lopez et al. 2016; McCallister et al. 2016). Indeed, the interaction of HSP70 (HSPA1) with membranes was diminished by exchanging portions of PS with phosphatidylcholine (PC) within liposomes (Arispe et al. 2004; Armijo et al. 2014). Additionally, HSPA8 (HSC70) was found associated with PS on the cytosolic side of endosomes during microautophagy (Sahu et al. 2011). Other negatively charged phospholipids also mediated the interaction of HSP70 with membranes, including palmitoyl-oleoyl phosphatidylglycerol (POPG) (Armijo et al. 2014; McCallister et al. 2016) and bis(monoacylglycero)phosphate (BMP), the latter being a major phospholipid of lysosome membranes (Kirkegaard et al. 2010; Mahalka et al. 2014). In addition, HSP70 associates with cardiolipin that is present in mitochondrial membranes (Mahalka et al. 2014). Indeed, mitochondria HSP70 (mtHSP70), also known as mortalin (HSPA9), interacts with membranes containing cardiolipin, particularly resembling the inner mitochondrial membranes (Dores-Silva et al. 2020a). Other studies detected the interaction of HSP70 with the glycosphingolipid Gb3 (Gehrmann et al. 2008) and sulfogalactosyl ceramide (Mamelak et al. 2001), which are also negatively charged. HSC70 (HSPA8) showed also high selectivity for PS in addition to low affinity for PC (Dores-Silva et al. 2021). In contrast with the preceding observations, bacterial HSP70, DnaK, interacted with lipid membranes without any phospholipid specificity (Lopez et al. 2016), suggesting that the ability of HSP70s to associate with membranes may be an ancient characteristic of these proteins, and phospholipid specificity was gained during evolution.

The distribution of phospholipids within the plasma membrane is asymmetric, with PC head exposed outside the cells and PS and phosphatidylethanolamine (PE) located within the cytosolic side of the membrane. This asymmetric distribution is maintained by a complex and energy-consuming mechanism directed at correcting the potential spontaneous flipping of lipids across the bilayer (Leventis and Grinstein 2010). Therefore, cytosolic HSP70

could interact with the negatively charged phospholipids within the inner side of the plasma membrane, a process that could be followed by lipid bilayer insertion. Such an event may allow the exposure of some protein regions on the cell surface. Indeed, studies by Multhoff showed that only partial regions of Hsp70 are displayed on the cell surface (Botzler et al. 1998). The amount of HSP70 inserted into the plasma membrane of tumor cells has been reported to be less than 15% of the total cellular concentration of this protein (Gehrmann et al. 2008). Thus, the question that emerges is why only a fraction of the very abundant HSP70 is associated with the plasma membrane. We have proposed that only substrate-free HSP70 is capable of translocating into the lipid bilayer (De Maio 2011). This assumption is based on the observation that HSP70 did not appear on the plasma membrane immediately after heat shock but rather after several hours of post heat stress recovery (Vega et al. 2008), perhaps because HSP70 is in excess with respect to heat-induced unfolded proteins at late times after the insult (Fig. 1). The same argument could be used to explain how the constitutive HSPA8, which is also very abundant in normal physiological conditions, is not ordinarily present on the cell surface, even though this protein has the capacity to interact with lipids (Arispe and De Maio 2000; Macazo and White 2014). Indeed, HSPA8 is likely primary associated with substrates, particularly nascent polypeptides, perhaps preventing membrane insertion. The exception is the binding of HSPA8 to PS within endosomes as part of the process of microautophagy (Sahu et al. 2011). Another argument is that HSP70 is present, almost exclusively, on the membranes of cancer cells because these transformed cells have a great excess of HSP70 with respect to non-cancer cells (Calderwood et al. 2006), which are likely in larger abundance with respect to potential cellular substrates.

Other HSP70s, such as HSPA5 (BIP, Grp78), have also been found associated with lipid membranes. HSPA5 was detected inserted into the plasma membrane of cancer cells (Suzuki et al. 1991; Delpino and Castelli 2002; Zhang et al. 2010, 2013). In addition, the protein was released outside cells (Delpino and Castelli 2002; Zhang et al. 2013). The plasma membrane insertion and extracellular export of HSPA5 were not very surprising since this protein is a resident of the ER. However, HSPA5 needs to overcome the ER retention signal (KDEL) to reach the cell surface/extracellular environment, which could be a consequence of ER stress (Zhang et al. 2013) or any additional factors. HSPA5 is unlikely to interact with the internal ER membrane because the phospholipid composition of this region does not support membrane insertion (Dores-Silva et al., 2020b). Several domains of HSPA5 have been proposed to be inserted into the plasma membrane, particularly the C-terminus end (Tsai et al. 2015; Tseng et al. 2019). The interaction of HSPA5 with artificial lipid bilayers (liposomes) has confirmed membrane insertion, displaying a high affinity for negatively charged phospholipids (Dores-Silva et al. 2020b). Both HSPA5 N-terminal and C-terminal domains could independently interact with phospholipid membranes, but not at the same levels as the full-length protein, suggesting that the two regions may be involved in membrane insertion (Dores-Silva et al. 2020b).

Another HSP70, HSPA9 (mtHsp70, mortalin), that is mainly located in the mitochondrial matrix was also found to associate with negatively charged membranes, in particular cardiolipin, that constitutes approximately 18% of the inner membrane and less than 1% of the outer membrane (Zinser et al. 1991). Studies using liposomes resembling the composition of both inner and outer mitochondrial membranes showed that, indeed, HSPA9 has selectivity for the inner membrane (Dores-Silva et al., 2020a). A very important

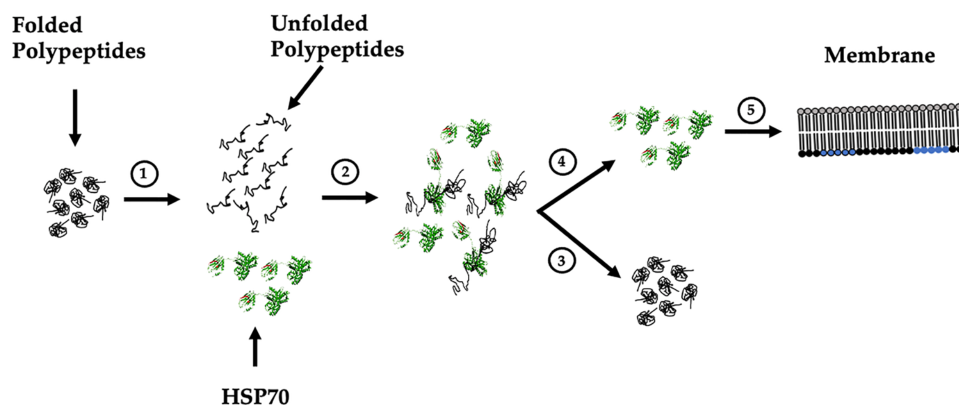

Fig. 1 Proposed mechanism for the translocation of HSP70 from the cytosol into the plasma membrane. Proteins are properly folding during normal physiological conditions that become unfolded upon heat shock (1) and the expression of HSP70. These newly expressed HSP70s bind to unfolded polypeptides (2), resulting in the refolding of denatured proteins (3) and an excess of polypeptide-free HSP70 (4), that is now capable of getting inserted into the plasma membrane (5) via the interaction with PS on the inner part of the bilayer

observation was the interaction of HSP70 with lysosome membranes specifically mediated by binding to the negatively charged phospholipid BMP that is a major component of this compartment (Kirkegaard et al. 2010; Mahalka et al. 2014). The association of HSP70 with lysosome membranes confers stability to this compartment preventing the leakage of lytic enzymes (Nylandsted et al. 2004). Moreover, the interaction of HSP70 and lysosomes appears particularly important in conditions of lysosome storage disorders, and it has been envisioned as a potential therapeutic target (Kirkegaard et al. 2010; 2016; Balogi et al. 2019).

Although all HSP70s displayed the same affinity for negatively charged phospholipids, their insertion into membranes is not identical. The interaction of HSPA1 and HSPA8 with lipids was different in a liposome aggregation assay, including differences in insertion kinetics and the effect of calcium and nucleotides (Arispe et al. 2002). Another example is the interaction of HSPA9 with POPS liposomes displaying a saturation profile that was not observed for HSPA1. Thus, the packing of the protein within the lipid bilayer or perhaps translocation into the lumen of the liposome appears to be different among these two HSP70 members (Dores-Silva et al., 2020a). Thermodynamic parameters measured during the insertion into artificial membranes indicated that the process is spontaneous but slightly different for HSPA1, HSPA5, HSPA8, and HSPA9, involving intramolecular interactions, Van der Waals forces, hydrophobic interactions, water displacements, and conformational changes (Dores-Silva et al., 2020a, b, 2021).

Other heat shock proteins also interact with membranes

Small HSP, which play a plethora of biological functions (Carra et al. 2017), have not escaped from the interaction with lipids. The small HSP of bacteria, Hsp17, was initially found sedimenting with membranes (Miyake et al. 1993), and it was later found to localize with the outer microbe membrane (Laskowska et al. 2004). Other studies have shown that alpha-crystallin (HSPB5) interacted with lipid membranes (Borchman and Tang 1996; Ifeanyi and Takemoto 1991). Moreover, this protein oligomerizes at higher temperatures driving the insertion into the membranes of vertebrate lenses (Tjondro et al. 2016). Interestingly, HSPB5 membrane association has been correlated with the development of cataracts (Boyle and Takemoto 1996; Cenedella and Fleschner 1992; Cobb and Petrash 2002). HSPB5 and Hsp17 have been reported to stabilize artificial membranes mediated by interaction with the polar head group and affecting the hydrophobic region of the lipid bilayer (Tsvetkova et al. 2002). Recently, HSPB1 and HSPB5 were found to get inserted into liposomes in which the alpha-crystallin domain characteristic of these proteins is embedded into the lipid bilayer. These two small HSP did not associate with the liposomes identically; neither did they display any phospholipid specificity (De Maio et al. 2019). These observations are similar to prior observations indicating that the interaction of HSPB5 with lipids was not specific for the type of phospholipids (Cobb and Petrash 2002) and was reduced by the presence of cholesterol within the membrane (Tang et al. 1998). HSPB5 has been found associated with a variety of membranes, including lenses (Boyle and Takemoto 1996; Cenedella and Fleschner 1992; Cobb and Petrash 2002; Friedrich and Truscott 2010), mitochondria (Whittaker et al. 2009), and Golgi (Gangalum et al. 2004; Gangalum and Bhat 2009). In addition, HSPH5 was observed participating in exosome assembly and release (Gangalum et al. 2016; Kore and Abraham 2016). Other HSP, such as Hsp90 (Hsp90B1), interacted with a mixture of phospholipids stabilizing the membrane (Li et al. 2018). Moreover, Hsp90 family proteins penetrated phospholipid membranes with high affinity losing part of their alpha-helix conformation (Li et al. 2019). In addition, Hsp90 (Hsp90A1) interacts with phospholipid membranes with higher affinity for unsaturated and negatively charged phospholipids, and the affinity increases in the presence of cholesterol (Zhang et al. 2018). GroESL oligomers also interacted with lipid membranes increasing their stability during heat shock conditions (Torok et al. 1997).

Mechanisms of heat shock proteins membrane insertion

The mechanisms for HSP membrane insertion are complex, poorly understood, and enigmatic, particularly because these proteins do not contain major hydrophobic domains that could explain their incorporation into lipid membranes. Biological membranes have a heterogeneous nature in which a hydrophobic center core is made by the assembly of fatty acid tails that are surrounded by a less hydrophobic environment constituted by the polar lipid heads, containing a fair amount of water that may create a niche for the initial insertion of proteins into membranes (Wiener, and White 1992). Thus, the interaction of proteins with the phospholipid head is likely the initiating event for membrane insertion that may be followed by a conformational change that facilitates the incorporation into the most hydrophobic region of the membrane, which may be part of or secondary to an oligomerization process (Wimley et al. 1998). Based on these assumptions, it is not surprising that HSP70 displays phospholipid head specificity and oligomerizes upon membrane insertion. Recently, the interaction of HSP70 with lipid membranes has been shown to result in a rearrangement of the hydration layer associated with the bilayer (Dhanasekaran et al. 2020).

Moreover, the insertion of HSP70s into the lipid membrane is a thermodynamically spontaneous process (Dores-Silva et al. 2020a; b; 2021). HSPA8 was reported binding to PS on the endosome membrane (Sahu et al. 2011), an interaction that was mediated by a cluster of lysine residues on the N-terminus end of the proteins, which was confirmed by site-directed mutagenesis (Morozova et al. 2016). This observation is consistent with increased interaction with PS liposomes at pH 5.0 and lower at pH 9.0 (Dores-Silva et al. 2021).

Prior studies have shown that HSP70 could form dimers and oligomers in solution (Guidon and Hightower 1986a; b; Benaroudj et al. 1996; Gao et al. 1996; Aprile et al. 2013), a process modulated by nucleotides (Kim et al. 1992; Benaroudj et al. 1996) or temperature (Angelidis et al. 1999; Kiraly et al. 2020). HSPA1 and HSPA9 in solution were observed as homogeneous round complexes of high molecular mass visualized by electron microscopy (Kiraly et al. 2020). Several models have been proposed for the oligomeric complexes, such as binding to the linker between the peptide and nucleotide-binding domains (Chang et al. 2008) and an antiparallel conformation (Morgner et al. 2015). Although no changes in HSP70 secondary conformation have been observed upon membrane insertion, oligomers of this protein have been detected upon incorporation into liposomes (Armijo et al. 2014; Dores-Silva et al. 2020a; 2021). Moreover, studies using atomic force microscopy showed the presence of HSP70 clusters on artificial lipid bilayers (Lamprecht et al. 2018). The best evidence of the oligomerization of HSP70s upon membrane insertion is its ability to form ion conductance channels (Arispe and De Maio 2000; Vega et al. 2008; Macazo and White 2014) which are assembled by various polypeptide subunits or multiple transmembrane domains. The oligomerization process upon membrane insertion may be enhanced by the fluidity of the bilayer as observed using phospholipids with different degrees of fatty acid saturation (Armijo et al. 2014; Lamprecht et al. 2018). Other saturated lipids such as sphingolipids have been reported to be recognized by HSP70 (Gehrmann et al. 2008; Mamelak et al. 2001). Interestingly, cancer cells display elevated levels of the glycosphingolipid Gb3 on the plasma membrane that could explain the presence of HSP70 on the surface of transformed cells (Gehrmann et al. 2008). In this regard, HSP70 have localized within lipid rafts that are rich in sphingolipids and cholesterol (Vega et al. 2008; Nimmervoll et al. 2015; Lamprecht et al. 2018).

As indicated above, HSPA1, HSPA5, and HSPA8 were observed to form oligomers after incorporation into lipid bilayers (Armijo et al. 2014; Dores-Silva et al. 2020a, b, 2021). These oligomeric complexes were stabilized via intermolecular disulfide bonds (Dores-Silva et al. 2020a). HSPA5 contains two cysteine groups, one at the beginning of the N-terminus end and the second at the C-terminus end. In contrast, HSPA1 presents five cysteine groups, with three at the N-terminus end and two at the C-terminus end. HSPA8 has four cysteine groups, two in the nucleotide-binding domain and two in the substrate-binding domain. One of the cysteine groups at the C-terminus end is the only common among all HSP70s. There is no evidence that these cysteine groups form intramolecular bridges in solution nor within the reducing cytosolic environment. Thus, it is possible that the lipid bilayer may provide an oxidative environment allowing the formation of disulfide bridges. Independent membrane insertion of the N-terminus end domain of HSPA1 and HSPA5 could form dimers but not high molecular mass oligomers that were only observed with the full-length protein, whereas membrane insertion of the C-terminus end did not form dimers or oligomers (Dores-Silva et al. 2020a). Based on these observations, we assume that a cysteine within the N-terminus end of the proteins may be within the right conformation to form dimers but not more complex forms. In contrast, we speculate that high-mass oligomers observed upon membrane insertion are the product of intermolecular disulfide bonds between the N-terminus end and the C-terminus domains of adjacent polypeptides assembling in an antiparallel conformation between tandem repeats (Fig. 2).

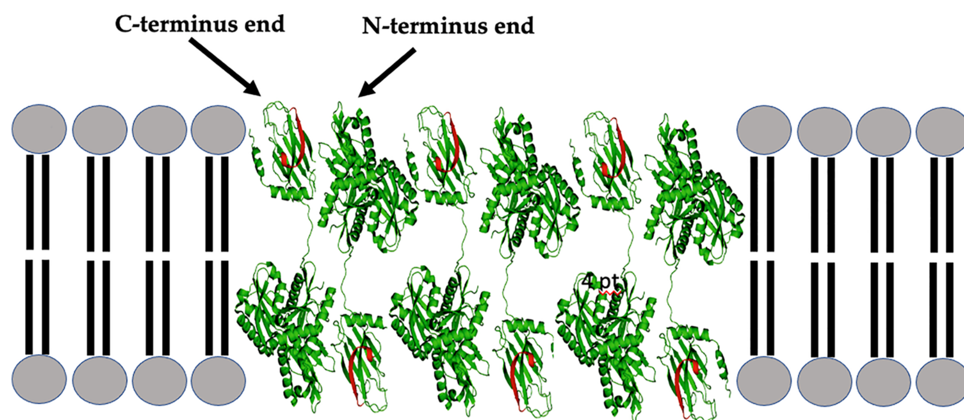

Fig. 2 Proposed model for the oligomerization of HSP70 within the lipid bilayer. HSP70 is assembled into the lipid bilayer in an antiparallel oligomeric complex in which the N-terminus end is bound to the C-terminus end via a disulfide bond

Why are heat shock proteins inserted into membranes?

The question that emerges is what is the function of HSP membrane association? There is extensive evidence from Vigh's group showing that HSP stabilize biological membranes (Horvath et al. 2008; Torok et al. 2014; Balogi et al. 2019), which they proposed as a major sensor for thermal stress due to disturbances in membrane fluidity (Csoboz et al. 2013; Balogh et al. 2013). Other studies have indicated lipid membrane stabilization by HSP90 (Li et al.2018, 2019) and HSP70 (Nylandsted et al. 2004). The presence of HSP70 on the surface of cancer cells may confer protection to these cells as well as provide an interface with the immune system (Botzler et al. 1996; Multhoff et al. 2020). Indeed, GRP78/HSPA5 has been implicated in tumor survival, proliferation, and resistance (Pfaffenbach and Lee 2011). In contrast to these observations, the insertion of HSP into membranes could be detrimental. Arispe et al. (2004) showed that exogenous addition of HSP70 could trigger cell death. This observation echoes prior studies showing that an intracellular excess of HSP70 was detrimental in the long term, even though that an early response was protective (Feder et al. 1992). These observations suggest that the potential cytotoxic effect of HSP70 requires that its expression is tightly regulated. Indeed, HSP70 half-life after stress is very short (Mizzen and Welch 1988). Moreover, HSP70 has been reported as a negative regulator of HSF-1, which is the master transcriptional factor for HSP expression (Gomez-Pastor et al. 2018). In addition, the translation of Hsp70 mRNA is reduced in cells that contain large amounts of HSP70 (Theodorakis et al. 1999). Additionally, Hsp70 mRNA has a very short half-life (approximately 1 h) after thermal stress (Theodorakis and Morimoto 1987), which was substantially reduced in cells already containing large amounts of HSP70 (Theodorakis et al. 1999). Also, changes in Hsp70 mRNA stability have been reported in various cell types (DiDomenico et al. 1982; Simcox et al. 1985; Petersen and Lindquist 1989; Ramos and Pastore 2001). In echoes of these observations, HSP70 was found bound to its own message (Balakrishnan and De Maio 2006), a situation that may be part of a mechanism for the self-limiting expression of this protein, as previously proposed (DiDomenico et al. 1982; De Maio 1999). Therefore, it will not be surprising to learn that interaction with membranes may be part of a regulatory mechanism.

HSC70/HSPA8 has been detected on endosome membranes participating in the microautophagy process (Sahu et al. 2011; Morozova et al. 2016). Expression of HSP70 upon heat shock and other stresses was found to increase the endocytosis of transferrin and its receptor (Vega et al. 2010). Moreover, HSP70 accelerates the phagocytotic process in macrophages (Vega and De Maio 2005). The interaction of HSP70s with subcellular vesicles may be necessary for the stabilization of these compartments as proposed for the interaction with lysosome membranes (Kirkegaard et al. 2010; Nylandsted et al. 2004). Moreover, the association of HSP70 with membranes and their intrinsic chaperone activity may raise the possibility that they could be membrane chaperones involved in the insertion of other proteins into membranes. Thus, HSP90, which does not display a significant binding to PS liposomes, was driven into these vesicles after co-incubation with HSPA8 (Dores-Silva et al. 2021). Therefore, HSPA8 may associate with HSP90 in solution prior to membrane association. However, whether HSP90 is inserted into the lipid bilayer or if it is peripherally bound to membrane HSPA8 is unknown. Interestingly, HSPA1 is also capable of bringing HSPA90 into membranes, but this ability is not shared by HSPA5 or HSPA9.

Another possible function for the presence of HSP70s on the plasma membrane may be related to a signal-transducing activity for receptors or co-receptors as proposed for GRP78/HSPA5 (Zhang et al. 2010). Interestingly, HSPA5 has been identified as a receptor for various viruses, including Borna disease (Honda et al. 2009), Coxsackie, dengue virus serotype 2, and Japanese encephalitis (Kottom et al. 2018). Recently, HSPA5 was proposed as an alternative site for the invasion of SARS-CoV-1 (Chu et al. 2018) and SARS-CoV-2 (Ibrahim et al. 2020), the latter being responsible for the COVID-19 pandemic. Latest evidence has shown that HSPA5 forms a complex with the angiotensin-converting enzyme 2 (ACE2) and SARS-CoV-2 spike protein (Carlos et al. 2021). Moreover, reducing surface HSPA5 diminished the membrane presence of ACE2, blocking viral entry. Moreover, HSPA5 displayed higher affinity for the spike protein of the new UK variant of SARS-CoV-2 (VUI202012/01) with respect to the original viral protein as indicated by in silico analysis (Elfiky and Ibrahim 2021).

HSP protein-membrane insertion could also be part of the extracellular export mechanism. With the exception of HSPA5 that is located within the ER, other HSP are present within the cytosol lacking the consensus signal for the classical secretory pathway. Indeed, Hightower and Guidon (1989) showed that the release of HSP70 from cells could not be blocked by classical secretory pathway inhibitors. This early observation was revisited by Hunter-Lavin et al. (2004), showing that indeed HSP70 was released from cells by a mechanism independent of cell death. However, a cloud was raised by Basu et al. (2000), indicating that cell lysis after necrosis was the source of circulating HSP70. Like many things in science, both reports were valid. De Maio and Vazquez (2013) described that HSP70 could be released by cell lysis as well as by the non-classical secretory

pathway. The preceding has been described for the export of several cytosolic proteins (Nickel and Seedorf 2008; De Maio 2011). The presence of HSP70 on the plasma membrane could allow this protein to be released via extracellular vesicles or exosomes. Indeed, HSP70s have been reported as a traditional component of exosomes (Lo Cicero et al. 2015). Moreover, HSP70 was found inserted into the exosome membrane (Gastpar et al. 2005; Vega et al. 2008; Gobbo et al. 2016; Chanteloup et al. 2020). In this regard, the formation of bleeds from artificial lipid membranes containing HSP70 was observed upon addition of cholesterol to the bilayer (Lamprecht et al. 2018). Cell surface HSP70 is localized with detergent-resistant membrane microdomains or lipid rafts (Vega et al. 2008; Gehrmann et al. 2008), which could be the precursor for the formation of exosomes (De Maio 2011). Another study has proposed that HSP70 is released associated with secretory-like granules (Evdonin et al. 2006). The insertion of HSP70 into the lysosome-endosome membrane could be an alternative mechanism for extracellular secretion (Nylandsted et al. 2004; Mambula and Calderwood 2006; Juhasz et al. 2013). Similarly, HSPB1 has also been proposed to be secreted via the endolysosome pathway (Rayner et al. 2008; 2009). Extracellular HSPs are capable of activating a variety of cellular responses that may be mediated by interaction with surface receptors. Indeed, several extracellular HSP binding proteins have been reported, including LRP/CD91, CD40, CD14, TLRs, c-type lectins, and Scavenger receptors, suggesting that there is not "a receptor" but a variety of binding partners (Calderwood et al. 2007b; De Maio 2014). Interestingly, Shevtsov et al. (2014) showed that exogenous HSP70 were captured by cells triggering the membrane translocation and subsequent export of endogenous HSP70. This observation supports the idea that an excess of subcellular HSP70 drives the appearance of this protein on the cell surface as described above (Fig. 1).

The proteotoxic and metabolic stress responses work against one another. Dai and colleagues proposed that this antagonism creates a third mechanism to balance cellular homeostasis (Dai, et al. 2015). Tezgin and coworkers have postulated that this new mechanism is actually the caloristasis network, in which HSF1 acts as a master proximal integrator (Tezgin et al. 2020). The term caloristasis was coined to pair with proteostasis, and like the latter, it emphasizes the integrative regulatory interactions by molecules like HSF1, which is necessary to understand cellular energy homeostasis in normal and stressed cells. Where to search for additional regulators is the question. One possibility is that the selective membrane association described above for HSP70 interactions with cardiolipin and the association of mortalin (HSPA9) with inner mitochondrial membranes could position these proteins toward the regulation of oxidative phosphorylation. There have suspicions about a connection between proteotoxic stress responses and downregulation of oxidative phosphorylation almost from the initial discovery of mortalin. Wadhwa and coworkers discussed that the yeast mitochondrial reduced form of nicotinamide adenine dinucleotide dehydrogenase (the initial electron acceptor complex of the mitochondrial electron transport chain leading to oxidative phosphorylation) was identified as a binding partner of mortalin (Wadhwa et al. 2002). Another connection comes through the NF-kB transcription family member RelA, also a mitochondrial binding partner of mortalin (Johnson et al. 2011). These same authors have suggested that tumor cells have become dependent on RelA for rapid growth and survival by virtue of its ability to change cells from oxidative phosphorylation to aerobic glycolysis. It is frequently said that tumor cells are "addicted" to HSP and that they have highjacked a normal defensive maneuver of stressed cells, the acquisition of cytoprotection. This defensive response involves conversion of energy transduction from oxidative phosphorylation to glycolysis to drive biosynthesis for the repair and replacement of damaged molecules, similar to why tumor cells are thought to switch to aerobic glycolysis to drive biosynthesis to support rapid proliferation, known as the Warburg Effect (Tezgin et al. 2020). Thus, mortalin could fulfill its role as a multifunctional integrator of caloristasis and proteostasis through its functions as a regulator of oxidative phosphorylation, as a central component of the mitochondrial protein import machinery, and as part of a damaged protein disaggregating complex (Iosefson et al. 2012). These observations echo early Ritossa's observations regarding other inducers of the stress response pointing toward mitochondrial energy production, particularly the electron transport chain.

The ability of HSP to interact with phospholipids and their capacity to stabilize membranes could have played a role during the evolution of cellular membranes from protocells to modern cells. Prebiotic fatty acids were likely to form small vesicles due to their amphiphilic nature in aqueous solutions that could encapsulate chemicals, forcing them to react, forming new compounds (Black and Blosser 2016; Damer and Deamer 2015). Thus, these vesicles containing a lipid bilayer are likely the precursor of protocells (Black and Blosser 2016; Segre et al. 2001). A key element for the size expansion from the protocell to more complex structures was the ability to stabilize the lipid bilayer. Elegant studies by Cornell et al. (2019) indicated that primitive membranes could be stabilized by the insertion of amino acids. Thus, the evolution of the protocell to advanced cells was likely mediated by the substitution of fatty acids with glycerophospholipids and amino acids with short peptides. These short peptides involved in membrane stabilization were likely to give rise to longer polypeptides retaining the membrane penetrating capacity. Therefore, proteins with membrane insertion and stabilizing abilities such as HSP may have played

an important role in the evolutionary progression of cells (De Maio and Hightower 2020). The capacity of ancestral HSP precursors for the interaction with lipid membranes was likely preserved during the evolution to modern chaperones. In other words, the ability of HSP to get incorporated into membranes was not discarded during the process of gaining new functions such as promoting protein folding. According to this hypothesis, ancient HSP were primary membrane-stabilizing proteins before they became chaperones.

Concluding remarks

The progress from the early initial observations of the association of HSP with fatty acids toward their detection on the cell surface, their insertion into artificial lipid bilayers, and our current understanding of the interaction of these proteins with membranes has been a remarkable journey, marked by a lot of controversies, but full of excitement. The mechanisms of membrane insertion and oligomerization have begun to be elucidated. The role of these proteins stabilizing membranes under stress conditions, their capabilities of sensing stress, modulating the movement of subcellular vesicles, their potential participation in cellular membrane biogenesis, and their role in several pathologies have created new excitement that is likely to increase in the upcoming years. However, it is clear that there is still more to be explored.

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