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Review

Membrane-associated stress proteins: More than simply chaperones

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ABSTRACT

The protein- and/or lipid-mediated association of chaperone proteins to membranes is a widespread phenomenon and implicated in a number of physiological and pathological events that were earlier partially or completely overlooked. A temporary association of certain HSPs with membranes can re-establish the fluidity and bilayer stability and thereby restore the membrane functionality during stress conditions. The fluidity and microdomain organization of membranes are decisive factors in the perception and transduction of stresses into signals that trigger the activation of specific HS genes. Conversely, the membrane association of HSPs may result in the inactivation of membrane-perturbing signals, thereby switch off the heat shock response. Interactions between certain HSPs and specific lipid microdomains ("rafts") might be a previously unrecognized means for the compartmentalization of HSPs to specific signaling platforms, where key signaling proteins are known to be concentrated. Any modulations of the membranes, especially the raft-lipid composition of the cells can alter the extracellular release and thus the immuno-stimulatory activity of certain HSPs. Reliable techniques, allowing mapping of the composition and dynamics of lipid microdomains and simultaneously the spatio-temporal localization of HSPs in and near the plasma membrane can provide suitable means with which to address fundamental questions, such as how HSPs are transported to and translocated through the plasma membrane. The possession of such information is critical if we are to target the membrane association principles of HSPs for successful drug development in most various diseases.

Contents

1. Introduction . . . 1654
2. Chaperones and their regulation . . . 1654
   2.1. Classification and basic functions of stress protein chaperones . . . 1654
   2.2. Regulation of chaperone gene expression: the membrane thermosensor theory . . . 1655
3. Overview of the membrane association of sHSPs . . . 1655
   3.1. Membrane association of sHSPs . . . 1655
   3.2. Membrane quality control by lipid–sHSP interactions . . . 1656
4. Membrane association of the GroEL/HSP60 family members . . . 1657
5. Membrane-associated stress proteins at work: the membrane expression of HSP70 and its release into the extracellular milieu by mammalian cells . . . 1658
   5.1. HSP70 surface membrane expression in mammalian cells . . . 1658
   5.2. Exosomal export of HSP70 from mammalian cells . . . 1658
   5.3. HSP70 membrane lipid interaction . . . 1659
6. HSP70 promotes cell survival by inhibiting lysosomal membrane permeabilization . . . 1659
7. Exploring HSP plasma membrane localization and translocation at the nanometer scale . . . 1659
   7.1. Classical methods . . . 1659
   7.2. Non-classical in vivo, in situ methods . . . 1660
8. Future prospects: membrane-associated HSPs as therapeutic targets . . . 1661
   Acknowledgements . . . 1661
   References . . . 1661

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1. Introduction

By subjecting the larvae of *Drosophila melanogaster* to temperature shock, Ritossa discovered in 1962 that high temperature induces a specific set of genes [1]. Later, the products of these genes were identified as "heat shock proteins" (HSPs) [2] and it was also shown that these HSPs can be induced in all species hitherto studied and they are among the most phylogenetically conserved proteins. Induced by a wide range of stressors, from temperature or UV stress to hypoxia, inflammation, infections or environmental pollutants, HSPs (also termed stress proteins) play an appreciable number of key roles in living systems. Their major classes are grouped according to their molecular weights: small HSPs (sHSPs), HSP60, HSP70, HSP90 and HSP100 [3].

Although the term "molecular chaperone" was used first by Laskey et al. in 1978 to describe the properties of nucleoplasmin [4], from the late 1980s it has come into general use to categorize a range of different proteins that share the common property of assisting the assembly of other proteins [3,5]. Many chaperones are heat shock proteins. The reason for this behaviour is that protein folding is severely affected by heat and, therefore, some chaperones act to repair the potential damage caused by misfolding. In fact, not all, but most of the HSPs function as molecular chaperones to guide conformational states critical in the folding, translocation and assembly of newly synthesized proteins. Although most newly synthesized proteins can fold in the absence of chaperones, a minority strictly requires them.

HSPs can regulate the life or death of cells directly, by modulating certain apoptotic signaling events, or indirectly, by participating in antigen processing [5,6]. Due to their multiple and vital functions, HSPs play a fundamental role in the pathology of a number of human diseases. Aberrantly high levels of certain HSP classes are characteristic in cancer cells, and the converse situation typically applies for type 2 diabetes or various neurodegenerative diseases.

HSPs reside not only in the cytosol, but also in the cellular organelles. Discussed as a central theme of this review article, a subpopulation of HSPs is present either on the surface or within the cellular membranes [7–10]. Via their specific membrane lipid interactions, sHSPs have been shown to modulate major attributes of the membrane lipid phase such as the fluidity, permeability, or non-bilayer propensity. It is emphasized that different HSPs have been found to associate to a variable extent with detergent-resistant microdomains ("rafts"), and the association of the HSPs with these microdomains can be modulated by stress [11]. The membrane microdomain-associated HSPs can evidently participate in the orchestration and activity of distinct raft-associated signaling platforms [12].

The mechanism involved in the membrane localization of HSP70, and its particular role in the immunotherapy of cancer cells, will also be discussed in detail in this paper.

The HSPs were long thought to be cytoplasmic proteins with functions restricted to the intracellular compartment. However, an increasing number of observations have revealed that they can "escape" from the cell interior and enter the extracellular space. Irrespective of their secretary routes (raft-, exosome- or secretary granule-mediated, etc.), these exogenous HSPs proteins are important mediators of intercellular signaling. Among others, the secreted stress proteins can elevate the stress resistance of other cells, signal tissue damage to the inflammatory cells and ultimately stimulate both the innate and the adaptive immunity [13]. In spite of the great potential significance in the development of therapeutic strategies, the mechanisms of both the membrane association and the release of HSPs into the extracellular milieu remain unknown. Here, we show that in many cells and tissues several HSPs, lacking the signal sequences are found associated with plasma- or organellar membranes or cross the plasma membrane.

2. Chaperones and their regulation

2.1. Classification and basic functions of stress protein chaperones

There are many different families of HSP chaperones, each family acting to aid protein folding in a different way. Below, we give a brief survey of the major classes of these proteins. Table 1 summarizes the molecular chaperone families and lists their basic functions.

The sHSPs belong in a family of 12–43 kDa proteins that can form large multimeric structures and display a wide range of cellular functions, including the endowment of cells with thermotolerance in vivo and the ability to act as molecular chaperones in vitro. The sHSPs coaggregate with aggregation-prone proteins for subsequent, efficient disaggregation. The release of substrate proteins from the transient sHSP reservoirs and their refolding require cooperation with ATP-dependent chaperone systems [14]. In the lens, the sHSP alpha-crystallin can behave as a chaperone-like protein sequestering unfolded proteins, and inhibiting subsequent aggregation and insolubilization thereby maintaining transparency [15]. Human HSP27 and mouse HSP25 form large oligomers

Table 1
Major heat shock proteins and their function

Approximate molecular weight	Prokaryotic proteins	Eukaryotic proteins	Function
10 kDa	GroES	HSP10	Co-chaperone of GroEL
Small HSPs, 15–30 kDa	GrpE, IbpAB, HSP17	αA,B-crystallin HSP25/27, HSPB family	Blocks aggregation; in eukaryotes is also involved in regulation of actin assembly/disassembly.
40 kDa	DnaJ	HSP40	DnaJ acts synergistically with DnaK and the other co-chaperone GrpE. Suppresses polypeptide aggregation, promoting protein folding. Facilitate protein translocation through intracellular compartments or protein secretion.
60 kDa	GroEL, 60 kDa antigen	HSP60 TCP1	All bind to partially folded polypeptides and assist correct folding. In eukaryotes they are active in the mitochondrion/chloroplast.
70 kDa	DnaK	The HSPA group of HSPs including HSP71, HSC70, HSP72, Grp78 (BiP), HSX70 found only in primates	Protein folding and unfolding, provides thermotolerance to cell. All: bind to extended polypeptides; prevent aggregation of unfolded peptides; dissociate some oligomers; bind ATP and show ATPase activity. Hsp70 is involved in regulation of HSF1 activity and the repression of heat shock protein gene transcription. Also prevents protein folding during post-translational import into the mitochondria/chloroplast.
90 kDa	HtpG	The HSPC group of HSPs including HSP90, Grp94	Prevent aggregation of re-folded peptide; correct assembly and folding of newly synthesized protein. Maintenance of steroid receptors and transcription factors. Hsp90 appears to be involved in maintaining the HSF1 monomeric state in non-stressful conditions; represents 1–2% of total protein.
100 kDa	ClpB, ClpA, ClpX	HSP104, HSP110	Tolerance of extreme temperature.

Although the most important members of each family are tabulated here, it should be noted that some species may express additional chaperones, co-chaperones, and heat shock proteins not listed. Additionally, many of these proteins may have multiple splice variants (Hsp90α and Hsp90β, for instance) or conflicts of nomenclature (Hsp72 is sometimes called Hsp70).

which can act as molecular chaperones in vitro and protect cells from heat shock and oxidative stress when overexpressed. HSP25/27 is additionally involved in actin polymerization/depolymerization [16].

HSP60 (GroEL in *Escherichia coli*) is an ATP-dependent (un)foldase. GroEL, together with its co-chaperone GroES, forms a complex in *E. coli*, this being the best-characterized large (~1 MDa) chaperone complex [17]. GroEL is a double-ring 14mer with a greasy hydrophobic patch at its opening; it is large enough to accommodate the native folding of 54-kDa GFP in its lumen. GroES is a single-ring heptamer that binds to GroEL in the presence of ATP or ADP. GroEL/GroES may not be able to undo previous aggregation, but it does compete in the pathway of misfolding and aggregation [18]. The HSP60 homologues in eukaryotes are in mitochondria and chloroplasts. In the cytoplasmic compartment there is a family of proteins, termed T-complex polypeptides (TCPs), which are loosely homologous with GroEL and form double-ring complexes similar to that of GroEL [19]. TCP ring complexes are less abundant in the cytoplasm than would appear necessary for general protein folding, so they may have a limited range of substrates.

HSP70 (DnaK in *E. coli*) is perhaps the best-characterized chaperone. The HSP70 proteins are aided by HSP40 proteins (DnaJ in *E. coli*), which increase the ATPase activity of the HSP70s. Although a precise mechanism has yet to be determined, it is known that the HSP70s have a high-affinity for unfolded proteins when bound to ADP, and a low-affinity when bound to ATP [20]. HSP70 also acts as mitochondrial and chloroplastic molecular chaperone in eukaryotes [21].

The molecular chaperone, HSP90, facilitates the maturation and/or activation of over 100 'client proteins' involved in signal transduction and transcriptional regulation and is necessary for viability in eukaryotes. Largely an enigma among the families of heat shock proteins, HSP90 is central to processes broadly ranging from cell cycle regulation to cellular transformation [22]. The bacterial HSP90 homologue HtpG is expressed at low levels and is non-essential [23]. It is an ATP-dependent holdase that plays a role in the binding and stabilization/regulation of steroid receptors and protein kinases. Each HSP90 has an ATP-binding domain, a middle domain and a dimerization domain. They were originally thought to clamp onto their substrate protein (also known as a "client protein") upon binding ATP. The structures recently published by Vaughan et al. [24] and Ali et al. [25] indicate that client proteins may bind externally to both the N-terminal and middle domains of HSP90. HSP90 may also require co-chaperones such as immunophilins, Sti1, p50 (Cdc37) and Aha1 and cooperate with the HSP70 chaperone system [26,27].

The HSP100 (the Clp family in *E. coli*) proteins have been studied both in vivo and in vitro for their ability to target and unfold misfolded proteins [28]. The proteins in the HSP100/Clp family form large hexameric structures with unfoldase activity in the presence of ATP [29]. These proteins are considered to function as chaperones by recessively threading client proteins through a small (20 Å) pore, thereby providing each client protein with a second chance to fold [30]. Some of these HSP100 chaperones, e. g. ClpA and ClpX, associate with the double-ringed tetradecameric serine protease ClpP; instead of catalyzing the refolding of client proteins, these complexes are responsible for the targeted destruction of tagged and misfolded proteins [31].

2.2. Regulation of chaperone gene expression: the membrane thermosensor theory

The appropriate regulation of the chaperone expression, together with the cellular localization of HSPs, is critical for the health of the cell. Accordingly, an understanding of the mechanism whereby cells can elicit an HSP response is of key importance. Under non-stress conditions, HSPs are expressed at low levels and maintain the monomeric heat shock factors (HSFs), (the principal one in vertebrate is called HSF1) in an inactive, repressed state. De-repression of HSFs occurs as a result of the titration of HSPs away from HSF, by the stress-induced formation of denatured proteins. The HSF then translocates into the nucleus, trimerizes, undergoes hyperphosphorylation and binds to HSE in *hsp* gene promoters, thereby leading to the subsequent expression of their proteins [32].

Mild and obviously more "physiological" forms of stress (typically fever-range hyperthermia), however, do not appear to be coupled to protein denaturation [33]. During the past decade, a new but not exclusive "thermosensor" model has evolved, which predicts the existence of membrane-associated stress sensing and signaling mechanisms from prokaryotes to mammalian cells. In line with this concept, mild stresses, or "membrane defects" caused by different disease states, are sensed by changes in the fluidity and microdomain structure of membranes, without inducing a protein-unfolding signal [34–40]. In favour of this model, the exposure of mammalian cells to various membrane fluidizers or drugs with the ability to interact with certain membrane lipids substantially modulates *hsp* expression without inducing protein-unfolding [41,42]. In recent years, our understanding of the structure–function relationship of biological membranes has undergone considerable change as our knowledge of lipid microdomains ("rafts") has expanded [7–9,39,40]. Since lipid microdomains have been widely shown to play important roles in the compartmentalization, modulation and integration of cell signaling [41–43], we suggested that these microdomains may additionally have an influential role in stress sensing and signaling [44,45].

3. Overview of the membrane association of sHSPs

3.1. Membrane association of sHSPs

An early finding, underlying the potential role of sHSPs in membrane localization, was that a subset of the 15-kDa HSPs of *E. coli* was observed to sediment with membranes [46]. This subset of proteins was later identified as IbpA and IbpB. In parallel, the ability of IbpA and IbpB to protect cells from heat and oxidative stress was demonstrated [47,48]. IbpA/B in *E. coli* grpE280 cells were reported to be localized predominantly in the outer membrane [49]. While the transcription of the *hsp*17 gene of a blue-green alga, *Synechocystis* PCC 6803, is strongly regulated by changes in the physical order of the membranes, most of the newly synthesized HSP17 is also associated with the thylakoid membranes [35]. Evidence for the physiological relevance of the HSP17 thylakoid association was presented by Lee et al. [50], who reported that inactivation of *hsp*17 results in a greatly reduced activity of photosynthetic oxygen evolution in heat-stressed blue-green algal cells. Independent studies with another cyanobacterium strain, *Synechococcus* PCC 7942, showed that the constitutive expression of HspA, the sHSP from the cyanobacterium *Synechococcus vulcanus,* confers cellular thermotolerance and greatly increases the thermostability of the photosystem II (PSII) electron transport system [51], which is the most thermolabile element of the photosynthesis [52]. HspA plays a role in preserving the integrity of thylakoid membranes under heat stress, using strains in which constitutive expression of HspA does not affect expression of other HSPs such as GroEL and DnaK [53]. The stabilization may be a result of direct or indirect association of small HSP with thylakoids as employing immunocytochemistry to localize HspA in the cell showed direct evidence for the association of HspA with thylakoids. HspA changes subcellular localization after heat shock as the association of HspA to thylakoids greatly increased after 15 min of high temperature exposure. The expression of another sHSP, named Lo18, from the lactic bacterium *Oenococcus oeni* has been shown to be induced by administration of the membrane fluidizer benzyl alcohol, similarly as for heat stress. It has further been demonstrated that a subset of Lo18 is localized in the membrane fraction, the actual level of its membrane association depending on the temperature upshift [54]. HSP16.3, a small HSP from *Mycobacterium tuberculosis*, originally identified as an immunodominant antigen, was found to be a major membrane protein [55]. Gene

knock-out studies indicated that HSP16.3 is required for *M. tuberculosis* to grow in macrophages [56].

Early observations supported the membrane localization of the sHSPs in eukaryotic systems. In the course of studying the subcellular localization of the members of the *Toxoplasma gondii* sHSP family, it emerged that *T. gondii* sHSPs are located in different compartments. HSP29, one of the 5 sHSPs, was found to be membrane-associated on immunostaining [57]. The particular differences in the immunostaining patterns suggested that the targets and functions of *T. gondii* sHSPs might be fundamentally different. The thylakoid association of sHSPs in the chloroplasts of heat-stressed plants was first described by Adamska and Kloppstech [58]. Experiments with the green alga *Chlamydomonas* revealed that elevated levels of sHSPs increase the resistance of thylakoids to light and heat damage [59]. In particular, the sHSPs appeared to protect the PSII electron transport system [60].

Several members of the small HSP family, present in mammalian cells, are also associated with membranes. HSPB2, expressed in heart and skeletal muscle, associates with the outer membrane of the mitochondria [61]. α-crystallin, the major protein component of the vertebrate lens, is thought to play a critical role in the maintenance of transparency through its ability to inhibit stress-induced protein aggregation. However, during aging an increased level of membrane binding of α-crystallin is an integral step in the pathogenesis of many forms of cataracts [62,63]. Both αA and αB-crystallin homopolymer complexes and also a reconstituted 3:1 heteromeric complex have been shown to bind to lens plasma membranes in a specific, saturable and partially irreversible manner that is both time and temperature dependent. The amount of α-crystallin that binds to the plasma membrane increases under acidic conditions, but is not affected at high ionic strength, suggesting that α-crystallin mainly binds to the fibre cell plasma membranes through hydrophobic interactions. It has been concluded that the membrane association of α-crystallin is closely related to a loss of transparency in the lens [62,63]. A missense mutation (R120G) of αB-crystallin has been linked to a familial form of desmin-related myopathy (DRM). It is noteworthy that both αB-crystallin and HSP27 exhibit an enhanced plasma membrane localization in the myotubes of dexamethasone-treated DRM patients [64].

3.2. Membrane quality control by lipid–sHSP interactions

In view of the widely documented presence of sHSPs in membranes, it was suggested that a subset of sHSPs functions in the cellular "stress management" by acting as membrane-stabilizing factors.

Whether different HSPs interact with membrane proteins, membrane lipids or both, remains to be explored. However, it was proved for some sHSPs, that they differentially interact with membrane lipids and the interaction influences membrane physical properties. The critical roles of sHSPs in controlling the physical state, bilayer stability and integrity of membranes via specific lipid interactions have basically been established in the case of the sHSP from *Synechocystis* PCC 6803, where most of the heat-induced HSP17 is associated with thylakoid membranes [35].

As evidenced by a combination of genetic and in vitro studies, HSP17 is able to play a fundamental role in membrane quality control and thereby potentially contribute to the maintenance of membrane integrity under stress conditions [65]. Overall, *Synechocystis* HSP17 possesses not only protein-protective activity, located either in the cytosol or in the membranes [35], but also a previously unrecognized ability to stabilize the lipid phase of the membranes [66].

The interactions between purified HSP17 and large unilamellar vesicles consisting of synthetic or cyanobacterial lipids strongly increase the membrane microviscosity [65]. This ability of HSP17 has been documented by measuring anisotropy changes, using a fluorescent membrane probe (1,6-diphenyl-1,3,5-hexatriene, DPH), and by monitoring wavenumber alterations of the CH_2 stretches of lipid acyl chains by Fourier transform infrared spectroscopy (FTIR) [65].

Two members of the family of small heat shock proteins (sHSP) (α-crystallin and *Synechocystis* HSP17) have stabilizing effects on model membranes formed of synthetic and cyanobacterial lipids. In anionic membranes of dimyristoylphosphatidylglycerol and dimyristoylphosphatidylserine, both HSP17 and α-crystallin strongly stabilize the liquid–crystalline state [66]. Evidence from infrared spectroscopy indicates that lipid–sHSP interactions are mediated by the polar headgroup region and that the proteins strongly affect the hydrophobic core. In membranes composed of the non-bilayer lipid dielaidoylphosphatidylethanolamine, DSC studies revealed that, even at an extremely low protein:lipid molar ratio, HSP17 strongly stabilizes the lamellar liquid–crystalline phase at the expense of the non-lamellar lipid phase (H_{II}) [66], which is known to disrupt membranes under severe heat stress. This suggests that sHSPs can modulate membrane lipid polymorphism. In membranes composed of monogalactosyldiacylglycerol and phosphatidylglycerol (both enriched with unsaturated fatty acids) isolated from *Synechocystis* thylakoids, HSP17 and α-crystallin increase the molecular order in the fluid-like state. The data show that the nature of sHsp–membrane interactions depends on the lipid composition and extent of lipid unsaturation, and that sHSPs can regulate membrane fluidity [65,66]. We infer from these results that the association between sHSPs and membranes may constitute a general mechanism that preserves membrane integrity during thermal fluctuations.

Similar to the observations on *E. coli* sHSP IbpB [67], we have shown that recombinant HSP17 forms stable complexes with denatured malate dehydrogenase and serves as a reservoir for the unfolded substrate, transferring it to the DnaK/DnaJ/GrpE and GroES/GroEL chaperone network for subsequent refolding [65]. Surprisingly, large unilamellar vesicles made of synthetic and cyanobacterial lipids prove to modulate this refolding process substantially [65].

HSP17 behaves as an amphitropic protein and plays a dual role: depending on its membrane or cytosolic location, it may function as a "membrane-stabilizing factor" and/or as a member of a multichaperone protein-folding network. The membrane association of sHSPs antagonizes the heat-induced hyperfluidization and is simultaneously able to prevent the formation of a membrane-disrupting non-bilayer lipid phase. Since the lipid specificity seen with HSP17 has also been documented for αB-crystallin [65], this implies that the membrane binding of small HSPs through specific HSP/lipid interactions may confine the location of the HSPs to one or more membrane lipid domains. In line with this assumption, a "heat shock lipid", the highly saturated monoglucosyldiacylglycerol (MGlcDG), has been identified in *Synechocystis*. Out of five thylakoid polar lipid classes tested, MGlcDG, rapidly formed under heat/light stress conditions, expressed the strongest interaction with HSP17 [68].

Missense mutations at 17 positions throughout the HSP17 protein and a C-terminal truncation of 5 amino acids were recently identified [69] and subsequent biochemical assays differentiated these mutants into two groups. The C-terminal truncation and 6 mutations in the α-crystallin domain destabilized the HSP17 oligomer and reduced the in vitro chaperone activity. In contrast, 3 mutations had little effect on the oligomer stability or chaperone activity in vitro, although they significantly decreased the sHSP function in vivo. These mutations were clustered in the N-terminus of HSP17, pointing to a previously unrecognized, important function for this evolutionarily variable domain. Furthermore, the fact that the N-terminal mutations were impaired in function in vivo, but active as chaperones in vitro, indicates that current biochemical assays do not adequately measure essential features of the sHSP mechanism of action [70].

We examined mutants in the N-terminal arm, L9P and Q16R, for altered interaction with lipid and thylakoid membranes and examined the effects of these mutations on thylakoid functions [71]. We found that both mutant proteins had dramatically altered membrane interaction properties. Whereas L9P showed strongly reduced binding to thylakoid fractions compared to controls, Q16R was almost exclusively membrane-associated. Both WT HSP17 and Q16R sedimented

with liposomes made of *Synechocystis* membrane lipids indicating that these two proteins bind to lipids. In this experiment, L9P was completely localized in the supernatant fraction strengthening our idea that thylakoid membrane–sHSP interaction is governed by lipids.

Applying monomolecular layers of *Synechocystis* lipids to further study the sHsp–lipid interaction also indicated that the strong association of Q16R HSP17 with membranes can be linked to a specific lipid constituent of *Synechocystis* membranes. Compared to WT and L9P, Q16R displayed the highest interaction with monolayer formed both from TPL and from each individual lipid class. On the other hand, by far the highest degree of insertion of Q16R protein was recorded with a negatively charged lipid, SQDG.

In Q16R cells a specific modification of the Photosystem II complex was observed, which accelerated plastoquinone binding to the Q_B site. In addition, the presence of Q16R dramatically reduced UV-B damage of PSII activity due to enhanced PSII repair. We suggest that these effects occur at least partly due to increased interaction of Q16R with SQDG in the PSII complex. Our findings further support the model that membrane association is a functional property of sHsps and suggest sHSPs as a possible biotechnological tool to enhance UV protection. When all point mutations of *Synechocystis* HSP17 either resulting in dissociation of oligomers or causing a reduced ability to dissociate were tested for their lipid interactions, it turned out that the membrane-interacting HSP17 species are mostly dimers. In favour of this finding, Zhang et al. have shown that the dissociation of the oligomers of benzyl alcohol-inducible HSP16.3 in *M. tuberculosis* is a prerequisite for its plasma membrane binding [72].

It was documented earlier that the deletion of *ibpA* and *ibpB* in *E. coli* did not produce a stress-sensitive mutant strain. However, overexpression of both genes increases the resistance to heat and superoxide stress [47,48]. Our recent efforts were aimed at discovering the "membrane phenotype" (i.e. the identification of cells possessing altered membrane permeability and fluidity characteristics) by comparison of the wild type with Δ*ibpAB E. coli* cells. It should be noted that a similar observation emerged from tests on lipid interactions and the subsequent fluidity changes caused by the sHSP, Lo18, of a lactic acid bacterium [73]. Lo18 interacts with phospholipids and the association of Lo18 with liposomes formed from purified total lipids of *O. oeni* membranes reduced membrane fluidity at elevated temperatures. Indeed by fluorescence anisotropy of DPH, the membrane rigidifying effect of Lo18 is observable above 33.8 °C. This phenomenon was explained by GP of Laurdan which showed that Lo18 maintained phospholipids in a higher lipid order. Lo18 can modulate membrane lipid properties by direct action on phospholipids. Nevertheless, it is not excluded that the protein could interact with the membranes at all the tested temperatures, but the effect on the phospholipids is only detectable for temperatures superior to 33.8 °C with this technique.

Taken together, the above data reinforce the hypothesis that a lipid-associated pool of sHSPs may play important roles in the protection of membranes under stress conditions. We postulate that the thermally controlled, lipid-mediated and transient association of sHSPs to specific membrane domains, widely documented on the scale from prokaryotes to mammalian cells, may also serve as part of a feedback mechanism in the regulation of heat shock genes.

4. Membrane association of the GroEL/HSP60 family members

The group I chaperonins, comprising the highly conserved GroEL from *E. coli*, are generally regarded as soluble proteins that function in the cytoplasm of prokaryotes and in the matrix compartment of mitochondria and chloroplasts. In contrast with this classical dogma, however, a number of reports suggest the existence of an additional, membrane-associated subpopulation of GroEL homologues. By means of immunogold cryothin-section EM and immunofluorescence, Newman and Crooke [74] documented that 16% of the labelled GroEL proteins in the *E. coli* cell were located in the membrane fraction. Moreover, the relative density of gold particles bound to GroEL was significantly higher in the membrane region than in the cytosol (2.98 versus 1.89). Clearly, a subset of *E. coli* GroEL is associated with the membrane under non-stressed conditions. In *Mycobacterium leprae* and *Coxiella burnetii*, a proportion of the GroEL chaperonins sediment out with the insoluble pellet following cell lysis [75,76]. The early finding that GroEL is localized in the cytoplasmic membrane in the photosynthetic prokaryote *Chromatium vinosum* indicated that membrane-associated chaperonins assist in the post-translational assembly of oligomeric proteins in the membrane [77]. Binding of a chloroplast 60 kDa HSP to the thylakoid membrane has also been suggested in *Vigna sinensis* [78]. Localization of the chaperonins in the thylakoid region has been demonstrated in the nitrogen-fixing cyanobacterium *Anabaena* PCC7120 [79]. In *Borrelia burgdorferi* HSP60, which is primarily involved in the processing of flagellin, was shown to be present in the soluble fractions and the Triton X-100 detergent-soluble membrane fraction at temperatures ranging from 20 to 37 °C, and the relative amount of HSP60 associated with the membrane increased with growth temperature [80]. The major cytoplasmic membrane protein of *Legionella pneumophila*, a genus common antigen which proved to be the member of the HSP60 family of heat shock proteins, induces protective immunity in a guinea pig model of Legionnaires' disease [81,82].

The rapid, non-lethal heat exposure of *Synechocystis* cells induces an enhancement of PSII thermotolerance in parallel with HSP synthesis and an increased molecular order of the thylakoids. The analysis of *Synechocystis* HSPs revealed that the two GroEL homologues (Cpn60 and GroEL) [83–85] are distributed both in the cytosol and in the highly purified thylakoid fractions [86]. The thermoprotection induced by heat adaptation, together with characteristic changes in the membrane physical state, seems to operate more effectively in the light than in the dark [87]. Since the Cpn60 deletion mutant of *Synechocystis* cells fully preserves its ability to stabilize thylakoid membranes to sublethal heat it was concluded that the heat-induced membrane association of GroEL, but not of Cpn60, is a necessary and sufficient condition for thermoadaptation of thylakoid function. Alternatively, the membrane-stabilizing effects of the two GroEL analogues, independently of their apparently differing chaperone properties [86], are interchangeable.

E. coli GroEL chaperonin also associates with lipid membranes [88]. The binding is apparently governed by the composition and physical state of the host bilayer. The GroEL–lipid interaction occurs almost exclusively in the liquid–crystalline ("fluid") state of the model membrane. GroEL binding increases the membrane physical order, especially in the polar head-group region of the lipids, as probed with different fluorophores. It was concluded that GroEL chaperonins have dual functions: (a) to assist the folding of both soluble and membrane-associated proteins, and (b) to rigidify and therefore stabilize lipid membranes during heat stress [88].

In a variety of mammalian cells and tissues most of the HSP60 (80–85%) is localized in the matrix compartment of the mitochondria. Highly specific immunogold electron microscopy employing six different monoclonal and polyclonal antibodies revealed that about 15–20% of the labelling due to HSP60 was seen at discrete extra-mitochondrial sites, namely, in the mitochondrial outer membrane, plasma membrane, endoplasmic reticulum and peroxisomes [89].

Both microbial and human HSPs can act as dominant antigens in numerous infectious and autoimmune diseases, such as atherosclerosis, inducing a strong immune-inflammatory response. Confocal laser scanning microscopy has revealed an increased level of HSP60 in the mitochondria and on the surface of heat-stressed living and fixed human umbilical venous endothelial cells as compared with unstressed cells [90]. Atomic force microscopy (AFM), developed as a sensitive surface-probe technique in biology, has confirmed the presence of HSP60 on the membrane of stressed cells at an even higher lateral resolution, through the detection of specific single molecule binding events between the monoclonal antibody AbII-13 tethered to AFM tips and HSP60 molecules on cells [90].

Acute injury in adult cardiac myocytes results in the movement of HSP60 to the plasma membrane [91,92]. Plasma membrane HSP60 is detectable on the cell surface by the use of both flow cytometry and confocal microscopy and was shown to localize to lipid rafts. The localization of HSP60 to the cell surface correlates with increased apoptosis, which is accompanied by the translocation of bax to the mitochondria. Although the total amount of HSP60 is the same, it is no longer present in the cytosol to complex with bax, and bax is free to move to the mitochondria. Hypoxia results in dissociation of the HSP60–bax complex, with the translocation of cytosolic HSP60 to the plasma membrane and bax to the mitochondria [91]. It has been speculated that the interaction between HSP60 and bax may be critical in preventing apoptosis in the normal cell. The release of HSP60 may activate the innate immune system, promoting a pro-inflammatory state, including an increase in TNF-alpha. Thus, the abnormal trafficking of HSP60 to the cell surface may be an early trigger for myocyte loss and the progression of heart failure. The surface presentation of HSP60 on the myocyte combined with serum antibodies to this protein may be one mechanism fuelling the downward spiral in heart failure. It is possible that membrane HSP60 may be recognized by macrophages and hence mark the myocyte for destruction [92]. Whether or not the translocation of HSP60 to the membrane stabilizes its structure and exerts a protective effect is unknown, as is the mechanism(s) controlling this translocation. HSP60 has also been demonstrated in the extracellular space and identified in the plasma of some individuals [92]. HSP60 is believed to be a "danger signal" to the immune system and also highly immunogenic. Thus, extracellular HSP60 is possibly toxic to the cell. In the cardiac myocytes, HSP60 is released via exosomes [92]; within the exosome, HSP60 is tightly attached to the exosome membrane (exosomes are internal vesicles of multi-vesicular bodies (MVBs) that are released into the extracellular milieu upon fusion of the MVBs with the cell surface). Lipid rafts are involved in this process, as the inhibition of lipid raft formation reduces the release of HSP60 [92]. Carbonate treatment does not remove HSP60, whereas proteinase K treatment does remove HSP60, while leaving the Na-K-ATPase intact. These results indicate that HSP60 is tightly bound to the surface of the membrane, rather than inserted into the membrane [92].

5. Membrane-associated stress proteins at work: the membrane expression of HSP70 and its release into the extracellular milieu by mammalian cells

5.1. HSP70 surface membrane expression in mammalian cells

HSPs with molecular weights of about 70 kDa are found in nearly all subcellular compartments, where they support the folding of nascent polypeptides, prevent protein aggregation, and assist the transport of other proteins across membranes [5]. Apart from the location in the cytosol, we determined a plasma membrane localization of HSP70, the major stress-inducible member of the HSP70 family on mammalian tumor cells, by the flow cytometry of viable tumor cells, and by selective cell surface iodination [93]. These findings are in line with published data, indicating an abundance of molecular chaperones, including HSP70, in the plasma membrane of human tumor cell lines, as measured by the global profiling of membrane-bound proteins [94]. Although the exact mechanisms underlying the transport of HSP70 from the cytosol to the plasma membrane remain to be elucidated, the major stress-inducible, cytosolic HSP70, which lacks a transmembrane domain, is released from various tumor cell types [95–98]. It is still a matter of debate whether HSP70 is secreted as a soluble protein or whether extracellular HSP70 is bound to liposomal vesicles. However, there is no doubt that extracellular located HSP70s have immunomodulatory capacities and are potent agents in the activation of the innate and adaptive immune system. On one hand, in response to soluble HSP70, monocytes secrete pro-inflammatory cytokines through a receptor-dependent signaling pathway [99,100]. In the absence of bound immunogenic peptides, HSPs act as non-specific "danger signals" for the immune system. C-type lectin receptors, including CD94, and members of the NKG2 family, scavenger receptors and Toll-like receptors (TLRs) are currently discussed as potential interaction partners for HSPs [12]. On the other hand, membrane-bound HSP70 has been identified as a target structure for the cytolytic attack mediated by natural killer (NK) cells. By using autologous tumor sublines with different HSP70 membrane expression patters, generated by antibody-based cell sorting, we demonstrated that tumor cells with a high amount of HSP70 on their cell membrane are killed significantly better by NK cell, as compared with their low-expressing counterparts [101]. Moreover, the incubation of NK cells with soluble HSP70 or with an HSP70 peptide (aa 450–463, TKDNNLLGRFELSG) in combination with a low dose of IL, further enhances the cytolytic activity of NK cells and stimulates the enhanced secretion of IFN-γ and granzyme B, a proapoptotic enzyme [102]. In contrast, CD3+ T lymphocytes do not respond to identical stimulation (Multhoff, unpublished observation).

As discussed above, members of the HSP70 family are known to be most efficient if they operate in concert with other HSPs, including DnaJ and HSP90 family members, and also with co-chaperones that dictate their function in distinct cellular compartments [103,104]. Members of the anti-apoptotic Bcl-2-associated athanogene (BAG) family are well-characterized partners for HSP70. For Bag-4, also termed the silencer of the death domain (SODD), an interaction with the ATPase domain of HSP70 has been documented.

5.2. Exosomal export of HSP70 from mammalian cells

Exosomes with a diameter of approximately 40–100 nm and a floating density of 1.17–1.19 g/ml correspond to internal multivesicular bodies (MVBs) and are secreted upon fusion with the plasma membrane [105]. Apart from professional antigen-presenting cells [106–108], T cells [109], reticulocytes [110,111], platelets [112], B cells [113] and mast cells [114] tumor cells [115,116] have been reported to have the capacity to release exosomes. In accord with these findings, we reported previously that HSP70 is actively released from viable tumor cells in detergent-soluble vesicles, which exert biophysical and biochemical characteristics of exosomes. It is worth mentioning that exosomes contain cytosolic proteins, but lack ER-residing proteins. On the surface, exosomes present tetraspanning proteins and frequently mimic the plasma membrane expression pattern of the tumor cells from which they were derived. For example, tumors presenting high amounts of HSP70 and Bag-4 on their plasma membrane also present these molecules on their exosomal surface, whereas tumors with low HSP70/Bag-4 plasma membrane expression levels release exosomes with low amounts of surface-bound HSP70 and Bag-4.

The capacity to release exosomes is cell type-specific, though the amount of exosomes secreted can be enhanced by exogenous stress [117]. Exosome formation has been found to be associated with the small GTPase Rab-4, a marker for a trafficking pathway between early endosomes and the plasma membrane [118]. Indeed, Rab-4 has proved to be enriched in tumor-derived exosomes. In contrast, Rab-11, a marker GTPase for trans Golgi to plasma membrane transport, Rab-7, typical for protein degradation and Rab-9, marking the retranslocation of proteins from late endosomes to trans Golgi, are not specifically enriched in tumor exosomes. Exosomes are produced by the inward budding of the endosomal membrane in a process sequestering particular proteins and lipids. It is conceivable therefore, that proteins present on the exosomal surface have the same topology as plasma membrane-bound proteins. A recent study highlighted the presence of lipid raft microdomains in exosomal membranes and suggested their participation in vesicle formation and structure.

With respect to the function of exosomes, different possibilities are under discussion at present. During reticulocyte maturation, the secretion of exosomes has been found to be associated with the loss of

transferrin receptors (TfRs). This export is coupled with the binding of TfR to HSC70. It is assumed that this release is a possibility for reticulocytes to externalize obsolete proteins [119]. On the other hand, exosomes secreted by antigen-presenting cells contain less TfR, but are enriched in immuno-stimulatory molecules, including the major histocompatibility complex (MHC) class I/II molecules, HSP70 and HSC70 [108,116]. This led us to hypothesize, that tumor-derived exosomes might have major implications in the intracellular communication of the immune system. It has already been reported that tumor-derived exosomes provide a source for shared tumor rejection antigens for the cross-priming of cytotoxic T lymphocytes [106]. Apart from this T cell-mediated immunity, tumor-derived HSP70/Bag-4-expressing exosomes induce a strong cytolytic and migratory capacity in NK cells towards HSP70 membrane-positive tumor cells [120,121]. In contrast, exosomes derived from HSP70/Bag-4 membrane low-expressing tumor cells fail to induce this NK cell activity. These data led us to hypothesize that NK cells might be activated and attracted by tumors in vivo via the secretion of exosomes presenting HSP70 on their lipid membrane. Mechanistically, the lysis of HSP70 membrane-positive tumor cells by exosome-activated NK cells has been identified as granzyme B-mediated apoptosis [122]. Granzyme B is an apoptosis-initiating enzyme which is produced by pre-activated NK and T effector cells.

5.3. HSP70 membrane lipid interaction

Although the immunological functions of membrane-bound and extracellular HSPs are obvious, the interactions of HSP70 with the plasma membrane and exosomal membranes remain to be elucidated. Since high salt and changes in the extracellular pH do not affect the HSP70 membrane expression pattern, it appears unlikely that HSP70 is bound to proteinous cell surface receptors. It was therefore assumed that HSP70 might associate directly with plasma membrane lipids, as suggested in 1989 by Hightower and Guidon [123]. Previous studies have revealed that members of the HSP70 family preferentially interact with artificial liposomes consisting of phosphatidylserine (PS) [124]. Biochemical and biophysical analyses have shown that the incorporation of HSP70s into lipid bilayers is followed by a transmembrane ion flow. Since this ion flow is stable, occurs in defined multilevel discrete electrical events, displays cationic selectivity and is ATP-dependent, the formation of a multiconductance cation channel has been assumed [125]. The presence of PS in the outer plasma membrane leaflet, as determined by calcium-dependent Annexin-V binding, serves as an early marker of apoptosis. Accordingly, it might be that the plasma membrane-bound HSP70 indicates that this cell will undergo programmed cell death. However, a comparison of the clonogenic cell survival [93], plating efficiency and proliferation rates has revealed that corresponding tumor cell types differing in HSP70 membrane expression pattern [101] do not differ. Thus, it might be that HSP70 is associated with other lipid components of the plasma membrane. Recent findings indicate that HSP70 is found in cholesterol-rich microdomains ("rafts"), defined as regions within the plasma membrane that are enriched not only in cholesterol, but also in glycosphingolipids, glycosylphosphatidylinositol (GPI)-anchored and acetylated proteins [126,127]. Lipid rafts [128], serve as assembly and sorting platforms for signal transduction complexes, increase cell interactions and enhance intercellular cross-talk. Most of these functions require the support of HSPs with chaperoning functions and may therefore be reasonable to assume that HSP70 is present in rafts. Although experimental evidence is accumulating in favour of the presence of HSP70 in lipid rafts, the final proof has still not been found. Detailed analyses of the lipid compositions of plasma membranes and exosomal surfaces of tumors with differential HSP70 membrane expression patterns are currently ongoing to identify further lipid compounds that enable HSP70 to interact with membranes.

6. HSP70 promotes cell survival by inhibiting lysosomal membrane permeabilization

HSP70 is localized to the membranes of the endosomal/lysosomal compartment of tumor cells, and it has been suggested that it can therefore inhibit lysosomal permeabilization induced by such diverse stimuli as cytokines, anticancer drugs, irradiation, oxidative stress and photolysis. Furthermore, the mere depletion of HSP70 from tumor cells triggers lysosomal membrane permeabilization (LMP), the release of lysosomal constituents into the cytosol, and cathepsin-mediated programmed cell death. Thus, the main mechanism by which HSP70 confers a survival advantage on tumor cells appears to be the inhibition of the permeabilization of lysosomal membranes and/or membranes of other vesicles containing cathepsins. This may explain the widely demonstrated ability of HSP70 to protect tumor cells against the diverse death stimuli that all trigger LMP, but may kill cells in a caspase-dependent or -independent manner [129–131]. Obviously, the subcellular localization of HSP70 appears to be crucial for its ability to inhibit LMP. This has been demonstrated by using the CX^+ and CX^- sublines of CX2 colon cancer cells, which differ from each other only in their membrane expression, but not in the total level of HSP70 [101]. As discussed above, the manner in which HSP70 is localized to the lysosomes and plasma membrane of tumor cells is still an open question. However, there is direct evidence for a specific, pH-dependent and high-affinity interaction between HSP70 and the lysosomal membrane lipid lysobisphosphatidic acid (LBPA) [132,133]. LBPA is a known lipid co-factor for the enzyme acid sphingomyelinase, and the binding of HSP70 to LBPA antagonizes directly its activity, providing an explanation for the cytoprotective effect of lysosome-associated HSP70. Interestingly, an antibody towards LBPA reverses the protective effect of HSP70, revealing a possible target for future cancer therapy.

7. Exploring HSP plasma membrane localization and translocation at the nanometer scale

7.1. Classical methods

Biochemical studies on the localization of HSPs to a specific organelles or membranes require fractionation of the cell followed by labelling with antibodies. Despite providing a wealth of information and otherwise proving very useful, these classical measurements do not clarify how and where HSPs are localized in the plasma membrane. This is due in part to the disrupting conditions in these in vitro experiments, as important adaptors may be lacking, but could be required for the true location and effect of membrane-localized HSPs in living cells.

A special case of fractionation is the observation of DRMs, which lead to the introduction of the concept of lipid rafts [128]. In Caco-2, a model of cultured human intestinal cells, the amount of HSP70 was selectively increased in DRMs. Manipulating the lipid composition of DRMs in these cells resulted in a concomitant modulation of HSP70 release, thus suggesting that lipid rafts may represent a cellular mechanism for membrane delivery and release of HSPs [11]. At the same time, the release of HSP70 in peripheral blood mononuclear cells has been found either to be dependent on DRMs [134] or independent of them [135]. In general, the findings of whether DRMs are involved in the binding to and release of HSPs from the plasma membrane must be assessed with caution, considering that Triton X-100 and other detergents used to isolate the DRMs, may itself induce the formation of ordered domains in previously homogeneous lipid membranes [136]. The above finding emphasizes the need for complementary methods that allow an in vivo, detailed and dynamic observation of the plasma membrane, as an obvious choice with which to explore the process of HSP localization and translocation.

Another method commonly used in combination with fluorescently tagged antibodies to characterize the extracellular, membrane-

associated localization of HSPs in living cells is fluorescence activated cell sorting (FACS). This leads, for example, to the establishment of membrane-associated and extracellularly accessible HSPs in various cells [137]. When analysis of the intracellular amount of HSP is desired, cells are fixed and permeabilized, again a procedure with the potential of influencing the association of HSPs.

Fortunately, fluorescence and other techniques have been developed to such an extent in recent years that they may now be used to address the questions of how HSPs reach the membrane, where and to which component of the membrane they are localized and how they are released. We describe here some of the novel technologies that can be extremely powerful to monitor HSP localization and translocation in living cells.

7.2. Non-classical in vivo, in situ methods

Confocal microscopy is routinely employed to monitor protein localization inside a cell and/or in the cell membrane of living cells. In consequence of the diffraction-limited resolution, classical confocal microscopy is not sufficient to define the precise location of "membrane-associated" proteins [138] (e. g. a pool of vesicles within ~100 nm of the membrane versus membrane inserted HSPs). Confocal microscopy was provided with higher resolution by Hell et al. through use of the concept of stimulated emission depletion (STED) to reduce the confocal volume [139]. This is achieved by applying a second, red-shifted laser beam to quench excited molecules at the rim of the confocal spot (both along the optical axis and in the radial direction), thereby leaving only the molecules in the centre to fluoresce [140]. In the imaging of synaptotagmin vesicles with STED-microscopy, a resolution of 45–66 nm was achieved, which is well in the range of the vesicle size of 35–40 nm as determined with electron microscopy. This optical high resolution revealed that synaptotagmin remains clustered after synaptic vesicle exocytosis [141]. With respect to HSP analogue experiments, it might be possible to visualize whether fluorescently tagged HSPs are in fact in the plasma membrane or simply associated with it, as "surface staining" of the plasma membrane is not necessarily a proof that a protein is in the plasma membrane, nor does it discriminate between associated vesicles and associated proteins. Total internal reflection fluorescence microscopy (TIRFM) illuminates a ~100 nm thin layer at the glass/buffer interface [142,143]. This results in the high contrast of fluorescent molecules in and close to the plasma membrane of a cell attached to a glass surface. As TIRFM illumination is carried out in wide-field, a large area can be imaged simultaneously, which permits ms to s time resolution. This makes TIRFM the method of choice to monitor molecules, dynamic processes near and in the plasma membrane, e. g. endo- and exocytosis [144–146]. With respect to HSP localization and release, TIRFM with its ability to visualize single vesicles near the plasma membrane might provide detailed insight as to how HSPs are transported to and released from the plasma membrane.

Single particle tracking (SPT) [147] and its single fluorophore analogue single dye tracing (SDT) [146,147] follow the diffusion of a tagged probe molecule in the plasma membrane with high positional accuracy of a few 10 nm. This degree of accuracy allows the probing of details of membrane structure and dynamics [148–150] and has revealed the unconfined diffusion of the GPI- anchored protein CD59 in T24 (ECV) cells, which was previously concluded to be confined [151]. The tracking of HSPs in live cell plasma membrane could provide analogous information on the details of molecular motion in live cells, which may in turn provide clues concerning possible HSP release mechanisms.

For exploration of the details of molecular association in the plasma membrane, the single molecule variant of fluorescence recovery after photobleaching is available. In the protocol termed "thinning out clusters while conserving the stoichiometry of labelling" (TOCCSL), for example the stoichiometry of molecular aggregates, "signaling platforms", in the cell membrane can be determined in live cells [152]. For HSPs, TOCCSL could provide a means of analysing the exact number of HSPs in a molecular aggregate (putative signaling platform) in the plasma membrane and its alteration in response to (heat) stress.

In fluorescence resonance energy transfer (FRET), energy is transferred from a fluorescent donor to an acceptor fluorophore upon donor excitation through a non-radiative mechanism with a strong distance dependence in the nm regime [153–155]. FRET has been used to explore the existence of DRMs by measuring the distribution of GPI-anchored proteins (GPI-APs) in live cell membranes. The results as to

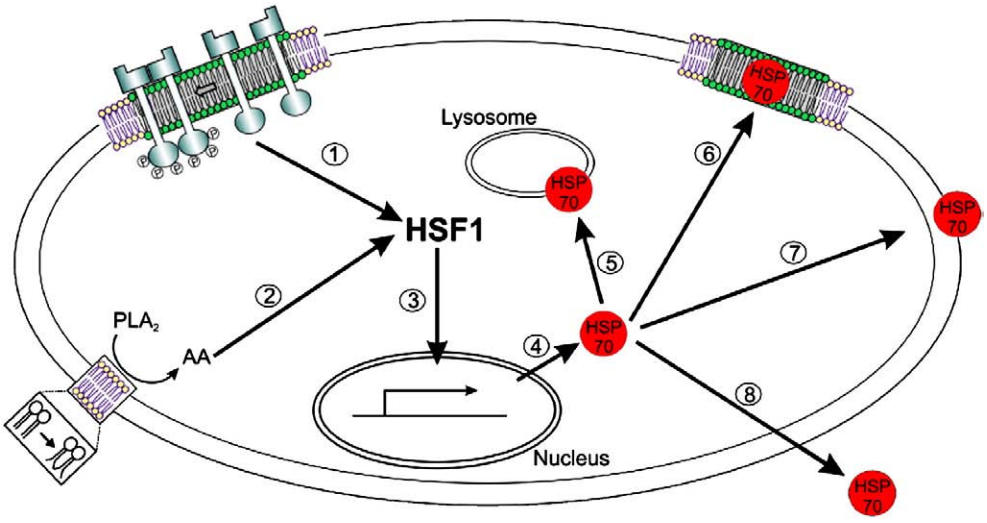

Fig. 1. Membrane mediated stress protein response and the cellular localization of HSP70 in mammalian cells. HSF1 is a key coordinator of the initiation of heat shock gene transcription which is activated by the appearance of denatured or misfolded proteins. In addition, a stress sensing-signaling mechanism operates through stress-induced membrane rearrangements. Such typical membrane mediated changes that trigger the expression of heat shock genes are the non-specific clustering of the growth factor receptors [164] associated with membrane microdomains ("rafts") (green coloured) (1) or the activation of phospholipase A_2 (PLA2) (2) [165], which sequesters itself into unsaturated-rich microdomains and cleaves arachidonic acid, which is a known HSP inducer. Stress activation of these pathways alters the nuclear accumulation and transcriptional activity of HSF1 (3) and ultimately modulates the actual level of HSPs, like HSP70 (4) [40]. The function of HSP70 depends on its intracellular, membrane-bound or extracellular location. The major action of chaperones in the cytosol is to maintain protein homeostasis. HSP70 can promote cell survival by inhibiting lysosomal membrane permeabilization via the interaction with the lipid LBPA (5). Experimental evidence is accumulating in favour of the presence of HSP70 in lipid rafts as component of signaling or trafficking platforms (6). HSP70 can also associate with lipids (like PS) in the plasma membrane and exhibit an immunogenic potential (7). Extracellular located HSP70s (8) have immunomodulatory capacities and are potent agents in the activation of the innate and adaptive immune system.

whether GPI-APs are or are not randomly distributed in cell membranes at present remain a matter of debate [156,157]. Because of the suspected small size of the interacting domains (possibly 5 nm or even less) [157] and the highly dynamic nature of the protein and lipid interactions in the plasma membrane, measurements of dynamic interactions of HSPs with lipids or membrane proteins on the molecular scale with FRET will be highly useful in the future.

Overall, it seems that exploration of the localization of HSPs in and near the plasma membrane on the nm scale at ms to s time resolution may provide the means with which to address important questions of what are the interactive partners of HSPs in the surface membrane and how HSP are translocated through the membrane.

8. Future prospects: membrane-associated HSPs as therapeutic targets

There are many aspects of HSP biology that remain puzzling, ranging from their roles under normal conditions to their functions in most various disease states. HSPs have been established to comprise a diverse group of essentially intracellular proteins that share the property of assisting the non-covalent assembly and/or disassembly of other macromolecular structures and are central components in many signal transduction pathways. Some chaperones were initially thought to form large complexes and were documented to act in a concerted way with a great number of co-chaperones in a "quasi-stoichiometric" manner.

It has subsequently become clear that this view is a major oversimplification. HSPs are present in almost all intracellular compartments, are also found on and within the endo- and surface membranes, and can be shed or secreted into the extracellular space (Fig. 1.). Accordingly, old dogmas relating to HSPs as therapeutic targets must also be reconsidered. Recent studies support the idea that the well-designed surface membrane and/or extracellular targeting of certain HSPs can enhance the immunogenicity of tumor cells, irrespective of their bulk level. Obviously, one approach with a beneficial clinical outcome could be the engineering of HSPs for higher secretion. On the other hand, the potential tools that are capable of inducing enhanced external HSP expression in malignant cells unambiguously point to membranes and membrane-interacting compounds as future therapeutic targets. The cell surface density of HSP70 on various tumor cells can be increased by non-lethal hyperthermia [157] or the administration of clinically safe reagents, ranging from phospholipase C inhibitors [158] to alkyl-lysophospholipids [159]. The lipid-interacting HSP co-inducer hydroxylamine derivatives [41–43], with having potential therapeutic value in the treatment of insulin resistance, heart disease or of certain neurodegenerative diseases have also the capability to increase the size of surface membrane-localized HSP70 in certain tumor cells (G. Multhoff, I. Horvath, I. Ando and L. Vigh, unpublished). We have documented that interactions between specific domains of membranes and certain HSPs remodel the pre-existing architecture and physical order of membranes. The highly specific HSP–lipid interaction can provide a hitherto unrecognized means of targeting HSPs to distinct compartments in the membrane, such as lipid rafts. Nevertheless, we feel it important to stress that much of the information concerning the degree of complexity of biomembranes has been largely overlooked until very recently. It may be concluded from the limited evidence available that certain diseases may be caused by minimal alterations to membrane hyperstructures [160]. For further research in this area, the non-invasive methods highlighted in this review article are essential, using living cells, and new techniques such as single molecule detection will undoubtedly prove invaluable. These methods, by exploring the localization of HSP in and near the plasma membrane at the nm scale at ms to s time resolution, will permit the mapping of diverse molecular interactions, for instance within membrane lipid molecular species and HSPs engaged in the control of membrane association and extracellular release of specific HSPs. Treatments, based on lipid therapy [39,161] which can restore the normal molecular interactions within membrane microdomains, and additionally rebalance HSP expression and distribution, offer new ways via which to protect against and alleviate a wide variety of human diseases and possess the potential to be of very widespread application in medicine [40].

Network theory is increasingly becoming a prevailing paradigm with which to describe the diverse cellular functions in both normal and disease or stressed states [162]. HSP chaperones are known to form complex networks (the "chaperome") with each other and their protein partners. As suggested by Csermely et al., modular analysis, novel centrality measures, the hierarchical representation of networks and the analysis of network dynamics will soon lead to a broadening of this field [163]. Obviously, this hierarchical cellular structure should be further extended by acknowledging that a subpopulation of HSPs is temporarily or permanently membrane-associated.

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