Characterization of the Relationship between the Chaperone and Lipid-Binding Functions of the 70-Kda Heat-Shock Protein, HspA1A

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Abstract

HspA1A is a molecular chaperone that plays indispensable roles in cellular survival. HspA1A also translocates to the plasma membrane (PM) of stressed and cancer cells. This translocation results in the cell-surface presentation of HspA1A rendering these tumors radiation insensitive. Thus, a putative therapeutic would be to inhibit HspA1A's PM translocation. However, to specifically stop the PM translocation of HspA1A, which is lipid-driven, it is imperative to characterize the lipid-binding regions of HspA1A and the relationship between the chaperone and lipid-binding functions of HspA1A, which remain unknown. To elucidate this relationship, we determined the effect of binding to phosphatidylserine (PS) on the secondary structure and chaperone functions of HspA1A. Circular dichroism revealed that binding to PS had minimal alterations on HspA1A's secondary structure. Measuring the release of inorganic phosphate revealed that PS-binding had no effect on the ATPase activity of HspA1A. In contrast, PS-binding showed subtle but consistent increases in the refolding activities of HspA1A. These observations strongly support the notion that the chaperone and lipid-binding activities of HspA1A are dependent but the regions mediating these functions do not overlap. These findings provide the basis for future interventions to inhibit HspA1A's PM-translocation in tumor cells, making them sensitive to radiation therapy.

Key words

chaperone function; heat-shock proteins; lipid binding; phosphatidylserine; protein refolding

Abbreviations

BMP: bis-(monoacylglycerol)-phosphate [sn-(3-oleoyl-2-hydroxy)-glycerol-1-phospho-sn-3'-(1'-oleoyl-2'-hydroxy)-glycerol)]; DPPC: 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; DPPS: 1,2-dipalmitoyl-sn-glycero-3-phosphoserine (sodium salt); Hsp70: Seventy-kilodalton heat shock protein; LVS: liposomal vesicle sedimentation; NBD: nucleotide-binding domain; PM: plasma membrane; POPC: Phosphatidylcholine [1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPS: Phosphatidylserine [1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (sodium salt); PS, Phosphatidylserine; SBD: substrate-binding domain; SGC: Sulfatide (3-O-Sulfo-D-Galactosyl-ß1-1'-N-Lignoceroyl-D-erythro-Sphingosine); SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis.

1. Introduction
HspA1A is a stress-inducible seventy-kilodalton heat shock protein (Hsp70) [1]. This protein is a molecular chaperone that plays critical roles in protein homeostasis and survival of stressed and cancer cells. In addition to its intracellular anti-apoptotic functions, HspA1A also localizes to the plasma membrane (PM) in 90% of human tumors resulting in radiation insensitive and metastatically aggressive tumors [2]. Thus, inhibiting HspA1A's PM translocation would augment current therapeutics and detail new ones.

Inhibition of HspA1A's PM localization requires prior knowledge of the molecular mechanism that drives this translocation. However, the mechanism that HspA1A uses to translocate to the PM is largely unknown, because the protein does not contain any known lipid-binding domains or other signals necessary for protein translocation to the PM. Nevertheless, multiple reports demonstrated that HspA1A binds to particular anionic lipids and further revealed that HspA1A's PM localization depends on its interaction with phosphatidylserine (PS) [3-6].

Therefore, inhibiting the binding of HspA1A to this lipid might stop the protein from localizing at the PM and subsequently HspA1A's cell-surface presentation. However, the regions of HspA1A that mediate the interaction with PS, as well as the relationship between the lipid-binding and chaperone functions, are largely unknown. This knowledge will provide critical information about the unknown regions of HspA1A that bind to PS, thus mediating the protein's PM localization, and allow us for future interventions to specifically target the lipid binding function of HspA1A as opposed to targeting the chaperone function, which has detrimental side effects.

To further characterize the interaction of HspA1A with PS and elucidate whether lipid-binding regulates its chaperone function, we determined the effect of lipids on the secondary structure and chaperone functions of HspA1A. To this end, we used two different lipids [phosphatidylcholine (PC) and PS] and two different lipid species for both lipids. We used both PC and PS because HspA1A binds with high affinity to PS but shows very low binding to PC [5-8]. Thus, these experiments would discriminate whether the presence of any lipid or lipid-specific binding is responsible for any observed change in HspA1A's chaperone activities. Moreover, PC is the most abundant lipid in mammalian cells [9] representing a lipid HspA1A would come in contact with during its cellular lifetime. Further, we used two different species of each lipid because it is known that HspA1A binds peripherally to liposomes composed of POPS, while it embeds in the lipid bilayer of liposomes composed of DPPS [5,8]. Thus, these experiments would discriminate between the effects of peripheral binding or embedding on chaperone function.

2. Materials and Methods
2.1 Lipids, Chemicals, and Reagents
All lipids were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Other common chemicals and reagents, e.g., antibiotics, buffers, and growth media, were obtained from Fisher Scientific or Sigma-Aldrich (St. Louis, MO).

2.2 Generation of recombinant clones, proteins, and protein purification
The mouse cDNA clone containing the *hspA1A* gene sequence, accession number BC054782 was used to generate the recombinant clones used in this study. The reaction conditions, primers,

and pet22b+ constructs as well as the protein production and purification are described in [5,7,10,11].

2.3 Generation of liposomes

Two different sizes of liposomes were generated in this study. Small unilamellar vesicles (SUVs) were generated by the freezing and thawing method as described in [5-7,12] and were used for assessing the lipid-binding properties of HspA1A because they are pelleted uniformly. To assess the effect of lipids on the structure, as well as the ATP hydrolysis and refolding activities of HspA1A, large unilamellar vesicles (LUVs) were used because of their higher stability. LUVs were generated using the extrusion method as described by Avanti Polar lipids (https://avantilipids.com/divisions/equipment-products/mini-extruder-extrusion-technique). The lipid composition and ratios used are provided in the figures.

2.4 Lipid binding assay

To quantify the lipid-binding of the different HspA1A recombinant protein batches the liposomal vesicle sedimentation (LVS) assay [5-7,12] was used. This method involves the quantitative pelleting of liposomal vesicles from a lipid/protein mixture. The extent of lipid-protein binding was then evaluated by comparing the amount of protein that remained in the supernatant to the amount of protein that was pelleted. The assay and the conditions used to assess the lipid-binding properties of HspA1A were performed as described in [5-7,12].

2.5 Circular dichroism (CD) spectra

To test the effects of lipids on the structure of recombinant HspA1A protein we used circular dichroism (CD) spectra analysis [13]. CD measurements were recorded on a Jasco J-810 Chirascan spectrophotometer (Applied Photophysics, Leatherhead, UK) with temperature controlled by a Peltier Type Control System PFD 425S. For these experiments, we used 1 mg/ml recombinant HspA1A protein in a 50 mM Phosphate Buffer (pH 7.4) at 25°C. Measurements were conducted in a 50 µL 0.1-mm pathlength Hellma Suprasil® Quartz cuvette (Sigma-Aldrich Catalogue Z805963). Spectra were recorded from 190 to 280 nm using a 0.1 nm step size with 0.5s time-per-point (with adaptive sampling), with each spectrum representing an average of three accumulations. Data of buffer and liposome suspensions without protein were also measured and their values were subtracted from the final protein spectra as background. Data were analyzed and plotted using the CD Analysis and Plotting Tool [14].

2.6 ATP hydrolysis assay

To test the effects of lipids on the function of HSPA1A, we determined whether the addition of liposomes affects the ability of HSPA1A to hydrolyze ATP. The ATPase assay used is a colorimetric assay that measures the amount of free inorganic phosphate. The assays were performed as described in [7,11,15] with the sole difference the incubation of HspA1A (1µM) with 1 mM of LUVs for 30 min at 30°C before the addition of ATP and the initiation of the reaction. This pre-incubation was performed because previous research has established that simultaneous incubation of ATP and lipid with HspA1A results in significant loss of lipid binding. In contrast, when the lipid and the protein are pre-incubated and the ATP is added later, the lipid-binding remains largely unchanged. The phosphate released in three independent experiments was plotted as the change of phosphate release over time [7,11,15].

2.7 β-galactosidase refolding assay

To test the effects of lipids on the refolding function of HSPA1A, we determined whether adding lipids alter the ability of HspA1A to refold denatured β-galactosidase [7,16]. To this end, β-galactosidase was chemically denatured and the recovery of the enzyme's activity relative to the activity of the native enzyme was measured after the addition of ATP and recombinant HspA1A. BSA and reactions with no enzyme were used as negative controls. The assay was performed as described in [7] and as in the ATPase assay, HspA1A (1μM) was pre-incubated with 1 mM of LUVs for 30 min at 30°C. The results were analyzed as described in [7] and plotted as percent of activity based on the activity of the native β-galactosidase. The refolding rate was calculated by using the linear regression line of all time points.

2.8 Statistical tests

Statistical significance was determined by One-way ANOVA with post-hoc Tukey HSD Test. A P value < 0.05 was considered statistically significant. The boxplots were generated using R software (http://shiny.chemgrid.org/boxplotr/) [17].

3. Results

3.1 HspA1A lipid-binding properties

HspA1A binds to specific anionic lipids, including different species of PS [3-6,8]. Between the different PS species tested, HspA1A embeds exclusively in liposomal membranes containing DPPS, while binds peripherally to less saturated species, i.e., DOPS and POPS [5,8]. Furthermore, the binding of HspA1A to different PC species, is relatively low and non-specific, and most likely not physiologically relevant [3-6,8].

 To ensure that the recombinant proteins used in the current study have similar lipid-binding characteristics as the ones described in the literature, we first used the lipid vesicle sedimentation (LVS) assay and quantified the binding of HspA1A to different species of both PC and PS. In agreement with the published literature, these experiments revealed that all three batches used in the current study bind to PS species significantly higher than to any of the PC species used (Fig. 1 and Supplementary Fig. 1). Furthermore, our results revealed that HspA1A binds to DPPS significantly higher than to POPS (Fig. 1B).
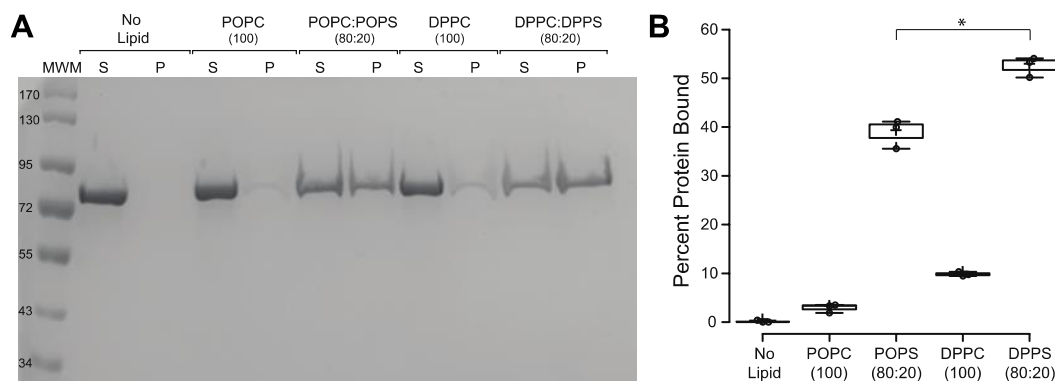

Fig. 1 HspA1A binds to liposomes containing phosphatidylserine. A single concentration of liposomes (1 mM total lipid) composed of different lipid mixtures in a specified molar ratio (see figure) was incubated with 1 μM of protein. After centrifugation, the extent of lipid-protein binding was determined by comparing the amount of protein that remained in the supernatant to the amount of protein that was pelleted. (A) Representative SDS-PAGE gel electrophoresis of the supernatant (S) and pellet (P) fractions of HspA1A with liposomes composed of different PC

and PS lipid species. (B) Quantification of the binding between HspA1A and lipids. The graphs are expressed as the percentage of protein bound to lipid vesicles (Y-axis). Error bars represent standard deviations for three independent experiments. The p-values for some of the comparisons were No lipid-POPC, p=0.308756; No lipid-DPPC, p=0.000308; POPS-DPPS, p=0.000153*.

3.2 Effects of lipids on the secondary structure of HspA1A

After verifying that the HspA1A recombinant proteins used in this study bind to lipids as expected, we sought to determine the effect of lipid binding on the secondary structure HspA1A. For this reason, we used the well-established technique Circular dichroism (CD) spectrometry [13]. In these experiments, we used single lipid liposomes (LUVs) composed of POPC (control), POPS (peripheral binding), and DPPS (embedding).

These experiments revealed that lipid binding caused only subtle changes in the secondary structure of HspA1A irrespective of which lipid was used (Fig. 2). Specifically, the presence of POPC caused a small increase and a subtle loss at the β-strand and α-helical regions of the protein, respectively. Differently, the presence of POPS, which HspA1A binds to peripherally, resulted in a small decrease of the β-strand and a subtle increase of the α-helical regions of the protein. Lastly, the presence of DPPS liposomes, which HspA1A binds and embeds, resulted in a small increase of the β-strand regions and a small reduction of the α-helical regions, similarly to POPC. However, these subtle changes were not consistent between the biological replicates and thus not significant (Fig. 2).
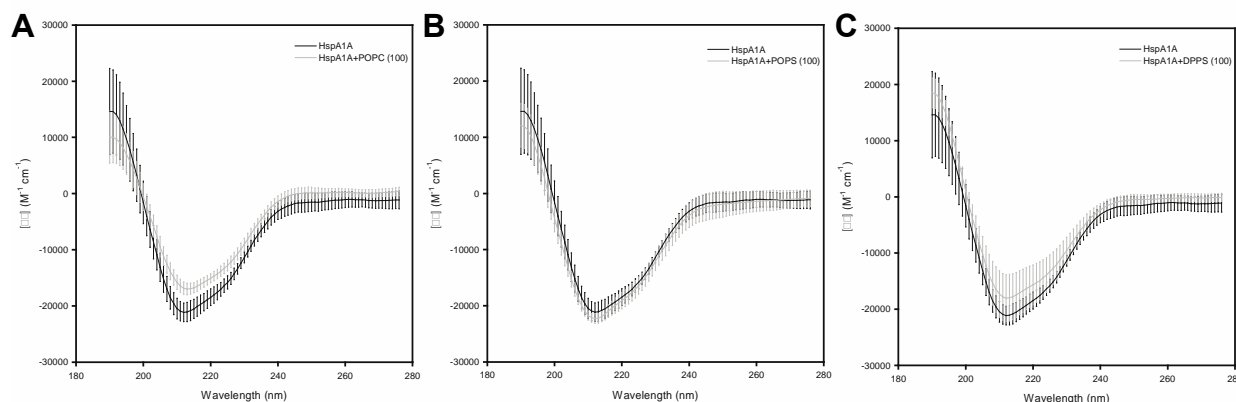

Fig. 2 Circular dichroism experiments reveal subtle alterations of the secondary structure of HspA1A in the presence of different lipids. Mean residue CD extinction coefficient values ($\Delta\varepsilon$ in M^{-1} cm^{-1}) were plotted and analyzed using the CD Analysis and Plotting Tool. Experiments were performed using three different protein batches, and the bars represent SD. The graphs compare the $\Delta\varepsilon$ values between HspA1A alone (black line) and HspA1A supplemented with (A) POPC, (B) POPS, and (C) DPPS liposomes.

3.3 Effects of lipids on the ATPase activity of HspA1A

HspA1A's binding to PS is mediated largely by the nucleotide-binding domain (NBD) of the protein [5,6]. Furthermore, binding to ATP, but not ATP hydrolysis, results in almost a complete loss of binding to PS, while this loss is not significant when the protein is pre-incubated with the lipid [5,6]. The latter finding suggests that although the conformational changes of HspA1A that are caused by ATP-binding are important for lipid binding, the regions responsible for nucleotide- and lipid-binding do not overlap. Based on these observations, we predicted that the presence and binding to lipids would change the ATPase activity of HspA1A.

To test this prediction and determine the effect of lipid binding to the ATPase function of HspA1A, we measured the release of inorganic phosphate at different time points in the presence or absence of liposomes (LUVs) composed of different lipids (Fig. 3). These results revealed that the presence of lipids results in a significant increase of released inorganic phosphate (Fig. 3). Specifically, both POPC and POPC:POPS, as well as DPPC and DPPC:DPPS liposomes, almost doubled the released phosphate. These results support the notion that the presence of lipids increases the ATP hydrolysis activity of HspA1A. However, this increase is induced at a similar level by either the control lipids (POPC, DPPC) or by the experimental lipids (POPS, DPPS) (Fig. 3). Therefore, these findings strongly suggest that the specific binding and embedding of HspA1A to POPS and DPPS do not specifically affect the ATPase activity of the chaperone.
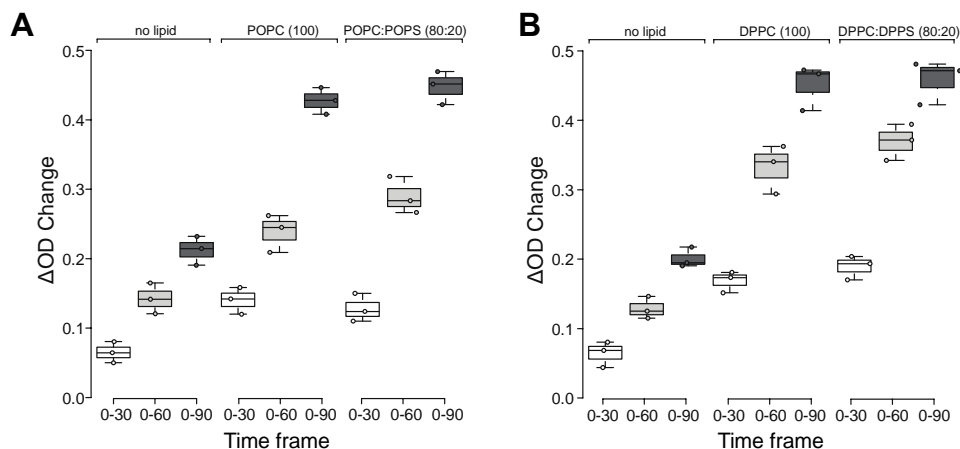

Fig. 3 The presence of lipids, but not lipid-binding, increases the ATP hydrolysis activity of HspA1A. The ATPase assay used is a colorimetric assay that measures the amount of free inorganic phosphate (Pi). Controls that contained no chaperone were used to count for spontaneous ATP hydrolysis, and their OD values were subtracted from the samples' values. The experiments were performed in the absence or presence of (A) POPC and POPC:POPS and (B) DPPC and DPPC:DPPS liposomes. The data are plotted as OD change (ΔOD) and are presented for three different periods (time frame). Each experiment was repeated three times. In all graphs, centerlines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles. The p-values of the statistical tests are provided in Supplementary Table 1.

3.4 Effects of lipids on the refolding activity of HspA1A

As noted above, HspA1A bind to DPPS primarily via the NBD region of the protein. In contrast, the substrate-binding domain (SBD) does not bind strongly to this lipid [5]. However, different reports revealed that the SBD is the region that actually embeds in liposomes composed of DPPS [5,8], suggesting that full-length HspA1A is necessary for the complete spectrum of the observed interactions. Based on these observations, we predicted the presence of DPPS liposomes, in which HspA1A embeds, will affect the refolding activity of the chaperone.

To test this prediction and determine the effect of lipid binding to the refolding activities of HspA1A, we measured the rate of refolding of chemically denatured beta-galactosidase in the presence or absence of liposomes (LUVs) composed of different lipids (Fig. 4). The results of these experiments revealed only small alterations of the HspA1A's refolding activities in the presence of lipids. Although the effect size of these changes is small, they seem to be specific, because BSA, the control protein used in this assay, did not show similar alterations (Fig. 4B).

Specifically, the presence of POPC, a control lipid that HspA1A binds non-specifically, did not significantly alter the total refolded enzyme. Differently, the presence of the other control lipid (DPPC), resulted in a small and consistent increase in the total refolded enzyme, although, the rate of refolding was not significantly different compared to the refolding observed in the absence of the lipid (Fig. 4C). The presence of POPS or DPPS, lipids that HspA1A bind, both revealed subtle but consistent increases in both the total refolded enzyme and the refolding rate (Fig. 4). These results suggest that binding to specific lipids slightly increases the refolding activity of HspA1A. However, the increase observed by both POPS and DPPS was very similar, suggesting that binding rather than embedding is the cause of increased refolding.
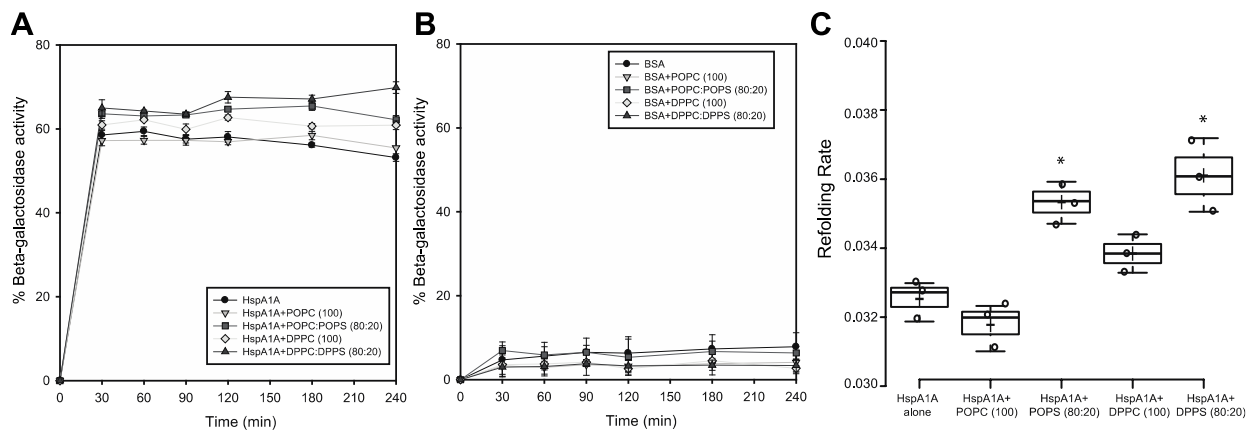
Fig. 4 The binding to phosphatidylserine results in a small but consistent increase of the refolding activity of HspA1A. The refolding assay used chemically denatured β-galactosidase and measured the recovery of the enzyme's activity by hydrolysis of ortho-nitrophenyl-β-galactoside relative to the activity of the native enzyme after addition of ATP, 1 μM recombinant HspA1A, and 1 mM of lipids (A). The data are plotted as percent of activity based on the activity of the native β-galactosidase. (B) BSA and reactions with no enzyme were used as negative controls. (C) The refolding rate was calculated by using the linear regression line of all time points. A p-value <0.05 is denoted with a *. The p-values of the statistical tests are provided in Supplementary Table 1.

4. Discussion

Recent research revealed that HspA1A translocates to the PM and is presented in the cell surface of multiple cancer types resulting in radiation insensitive and aggressive tumors. Therefore, several investigators aim to target the cell surface/PM-localized HspA1A as a novel anti-cancer treatment [2,18-20]. It is also fairly well established that HspA1A's PM localization depends to its interaction with intracellular PS and other lipids [3,4]. However, how HspA1A binds to PS remains a mystery because this protein does not contain any known lipid-binding domains or other PM translocation signals. Thus, if we manage to inhibit HspA1A's interaction with PS, we might be able to augment current therapies by stopping the protein from translocating to the PM while still maintaining its chaperone functions.

In this report, our goal was to advance our understanding of the HspA1A regions that bind to PS by determining whether the regions responsible for the chaperone functions of HspA1A overlap with the lipid-binding regions.

Our experiments verified that the proteins bind to lipids as expected and provided an important new finding that HspA1A binds significantly higher to saturated PS (DPPS) as compared to mono-saturated PS (POPS). This finding suggests that in addition to the ability of HspA1A to embed exclusively to DPPS containing liposomes, the protein also has a higher

affinity for saturated PS species. These observations might have important physiological implications because several well-controlled lipidomics studies revealed an increase in saturated lipid species in stressed and cancer cells [21].

We next assessed whether binding to or embedding into the lipid bilayer of liposomes containing PS alters the secondary structure of HspA1A using CD spectrometry. Our results strongly suggest that lipid binding and embedding do not cause major and specific alterations of the protein structure. These findings agree with results obtained using bis-(monoacylglycerol)-phosphate (BMP; [22]) and strongly suggest that although lipids might alter the conformation of HspA1A [5] they do not seem to affect the overall protein structure. Furthermore, these conformational changes are very small compared to the binding of nucleotides in the presence of Mg^{2+} [23].

Our next set of experiments determined that the addition of liposomes results in a significant increase of in ATP hydrolysis. However, all lipids used similarly induced these changes, irrespective of whether HspA1A binds them with high affinity or not. These observations suggest that binding to lipids or embedding in the lipid bilayers does not specifically affect the ATPase activity of HspA1A. This finding further implies that although in structural proximity [13], the regions that mediate ATP binding and hydrolysis and lipid binding do not overlap. These ideas are in agreement with previous research showing that the addition of ATP lowers lipid binding to background levels, but this loss-of-binding is less pronounced when HspA1A and lipids are pre-incubated prior to the addition of ATP [5,6,12]. Similar results were observed for another molecular chaperone (Hsp90), which also binds to lipids, although this study did not assess whether this increase is the result of specific lipid-binding [24]. Another lipid, SGC, induces oligomerization of HspA1A by locking it a high-affinity state to unfolded proteins and blocking the binding to ATP [25]. However, it is not clear whether this was the result of specific binding or if this was due to the presence of the lipid. Together these observations allow us to speculate that the presence of lipids that HspA1A interacts with, even with low affinities (e.g., PC, the most abundant phospholipid in mammalian cells), might stabilize the protein thus resulting in higher ATP hydrolysis.

Our last set of experiments determined that the addition of liposomes has only small effects on the refolding activity of HspA1A. These changes seem to be specific and clearly relate the refolding rate of the chaperone to the binding to specific lipids (i.e., POPS and DPPS). Nevertheless, the effect size of these changes (less than one-fold maximal increase) is relatively small and might not be physiologically important. This notion further strengthens our prediction that the regions of the protein responsible for lipid binding and refolding do not overlap. As in the case of ATP hydrolysis, we speculate that lipid binding might stabilize the protein and not directly interfere with its refolding abilities.

In conclusion, our results reveal that similarly to Hsp90, in which the membrane-deforming and chaperone functions are separated [26], the regions mediating the chaperone and lipid binding functions of HspA1A do not overlap. This knowledge will allow us to specifically target the lipid-binding function of HspA1A in order to inhibit the lipid-driven translocation of HspA1A to the cell surface of tumor cells, making them sensitive to radiation therapy.

Acknowledgments

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