



# The small heat shock proteins, HSPB1 and HSPB5, interact differently with lipid membranes

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## Abstract

Increasing evidence shows that heat shock proteins (hsp) escape the cytosol gaining access to the extracellular environment, acting as signaling agents. Since the majority of these proteins lack the information necessary for their export via the classical secretory pathway, attention has been focused on alternative releasing mechanisms. Crossing the plasma membrane is a major obstacle to the secretion of a cytosolic protein into the extracellular milieu. Several mechanisms have been proposed, including direct interaction with the plasma membrane or their release within extracellular vesicles (ECV). HSPB1 (Hsp27), which belongs to the small hsp family, was detected within the membrane of ECV released from stressed HepG2 cells. To further investigate this finding, we studied the interaction of HSPB1 with lipid membranes using liposomes. We found that HSPB1 interacted with liposomes made of palmitoyl oleoyl phosphatidylserine (POPS), palmitoyl oleoyl phosphatidylcholine (POPC), and palmitoyl oleoyl phosphatidylglycerol (POPG), with different characteristics. Another member of the small hsp family, HSPB5 ( $\alpha$ B-crystallin), has also been detected within ECV released from HeLa cells transfected with this gene. This protein was found to interact with liposomes as well, but differently than HSPB1. To address the regions interacting with the membrane, proteoliposomes were digested with proteinase K and the protected domains within the liposomes were identified by mass spectroscopy. We observed that large parts of HSPB1 and HSPB5 were embedded within the liposomes, particularly the alpha-crystallin domain. These observations suggest that the interaction with lipid membranes may be part of the mechanisms of export of these proteins.

**Keywords** Heat shock proteins · Exosomes · Membrane · Phospholipids · Liposomes · Stress

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## Introduction

Cells are constantly exposed to changes in their environmental conditions modulating their metabolism that could, in some circumstances, compromise homeostasis. Part of the cellular responses for conditions that depart from normal physiological conditions is the expression of heat shock or stress proteins (hsp). The activity of these proteins is directed at preserving basic cellular functions by a variety of mechanisms, including their ability to stabilize and renature unfolded polypeptides. Hsp consist of a large number of different polypeptides encoded by various genes, some of which are expressed during normal physiological conditions whereas others are exclusively activated after stress (De Maio 1999; Hartl and Hayer-Hartl 2009). One family of hsp corresponds to small molecular mass polypeptides, generally referred to as small hsp (Arrigo 2017; Carra et al. 2017), which are ATP-independent molecular chaperones (Jakob et al. 1993). The family of small hsp is made up of a variety of different polypeptides; the number of which varies greatly among different species (Boelens and de Jong 1995). A common feature of all of them is the presence of a domain coined the “alpha-crystallin domain” (Boelens 2014; Carra et al. 2017). This domain can form dimeric structures and facilitates the formation of large dynamic oligomeric structures that are characteristic for small hsp. In humans, ten different small hsp have been identified, named HSPB1 to HSPB10 (Kampinga et al. 2009), which show various patterns of expression (Boelens 2014).

HSPB1 (human HSP27) is constitutively expressed in several different cell types, but its levels are dramatically increased after heat shock (Arrigo 2017). The human protein has three phosphorylation sites, located at serine residues 15, 78, and 82. The level of phosphorylation at these sites plays an important role in many of their cellular functions, from protein folding to cytoskeletal organization and antiapoptotic and antioxidant properties (Arrigo 2017). In addition, HSPB1 displays varying stages of oligomerization from monomers and dimers to high molecular weight complexes above 800 kDa that are associated with various degrees of phosphorylation (Arrigo 2017). HSPB1 is also overexpressed in different tumors (Ciocca et al. 2015) and has been associated with other pathologies such as amyotrophic lateral sclerosis, Charcot-Marie-Tooth disease, and Alzheimer’s disease (Arrigo 2017). Another member of the small hsp family, HSPB5 ( $\alpha$ B-crystallin), is present in a variety of tissues, including the eye lens, heart, and brain. In particular, HSPB5 has been detected at high levels in various neurodegenerative diseases, including Alzheimer’s and Parkinson diseases and amyotrophic lateral sclerosis (Boelens 2014). Indeed, HSPB5 was found to block amyloid- $\beta$  aggregation and toxicity (Wilhelmus et al. 2006). HSPB5 can be phosphorylated at serine residues 19, 45, and 59, which reduces the size of

complexes (Peschek et al. 2013), modulates its cellular localization (den Engelsman et al. 2005), and affects the chaperone activity (Ahmad et al. 2008; Ecroyd et al. 2007). HSPB5 is also modified by the addition of O-GlcNAc groups (Roquemore et al. 1992), which affects its subcellular localization during heat shock (Krishnamoorthy et al. 2013).

Small hsp have also been detected outside cells (Batulan et al. 2016; Reddy et al. 2018), which echoes many other members of the hsp family (De Maio 2011). HSPB1 was first detected in the extracellular space of tumor cells (Banerjee et al. 2011; Feng et al. 2005) and subsequently in serum obtained from individuals suffering from several pathologies (Batulan et al. 2016; Reddy et al. 2018). Extracellular HSPB1 has been implicated in the activation of inflammatory responses (Batulan et al. 2016; Shi et al. 2019). In addition, the presence of HSPB1 in circulating fluids such as blood has emerged as an important biomarker of disease (Singh et al. 2017). HSPB1, as well as other hsp, do not contain a consensus signal for their export via the ER-Golgi secretory pathway. Therefore, these proteins could be released by passive mechanisms, including cell lysis and necrosis, or active processes independent of cell death (De Maio and Vazquez 2013). The latter has been coined the non-classical secretory pathway (Nickel and Seedorf 2008). For the translocation of a protein from the cytosol into the extracellular space, polypeptides need to cross the hydrophobic barrier made up of the plasma membrane. Several mechanisms have been proposed for the non-classical secretory pathway, including the transport across membrane pores and the release associated with extracellular vesicles (ECV) or exosomes (De Maio 2011; Nickel and Seedorf 2008). HSPB1 has been detected within the lysosomal compartment, opening the possibility that this protein is exported via the endo-lysosomal pathway (Rayner et al. 2008, 2009). HSPB1 has also been found associated with exosomes (Clayton et al. 2005; Nafar et al. 2016; Stope et al. 2017), which are vesicles released by active mechanisms upon the formation of multivesicular bodies or by direct blebbing from the plasma membrane (ectosomes); all commonly referred to as extracellular vesicles (ECV) (De Maio 2011; They et al. 2009). Another possibility is that HSPB1 could pass directly across lipid membranes. Certainly, some hsp, particularly members of the Hsp70 family, have been reported to associate with lipid membranes (Arispe et al. 2002; Armijo et al. 2014), and even opening ion conductance pathways (Arispe and De Maio 2000; Vega et al. 2008). HSPB5 has been reported also to associate with membranes, such as lens membranes (Boyle and Takemoto 1996; Cenedella and Fleschner 1992; Cobb and Petrash 2002; Friedrich and Truscott 2010), mitochondrial membranes (Whittaker et al. 2009), and Golgi membranes (Gangalum and Bhat 2009; Gangalum et al. 2004). In addition, this protein was detected within exosomes, playing an important role in the formation and release of these ECV (Gangalum et al. 2016).

Phosphorylation of HSPB5 was found to reduce exosome-mediated secretion, while an opposite effect was seen by O-GlcNAcylation (Kore and Abraham 2016). Based on these preceding observations, we investigated the ability of HSPB1 and HSPB5 to associate with lipid membranes.

## Material and methods

### Cell culture and isolation of ECV

HepG2 cells, a human hepatoblastoma cell line, were maintained in Eagle's minimal essential medium supplemented with nonessential amino acids (1×), sodium pyruvate (2 mM), L-glutamine (2 mM), penicillin (10 IU/mL), streptomycin (10 g/mL), and heat-inactivated fetal calf serum (10%). T-Rex™ HeLa cells expressing the Tet repressor (Invitrogen) transfected with pcDNA4/TO (Invitrogen) containing the coding sequences for wild-type HSPB5, HSPB5 S19D/S45D/S59D, and HSPB5 R120G (den Engelsman et al. 2004) were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, San Diego, CA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; PAA Laboratories, Linz, Austria), 100 U/mL penicillin, and 200 µg/mL streptomycin. Cells were maintained at 37 °C with 5% CO<sub>2</sub> in a humidified incubator. HepG2 cells (90% confluent, 150-cm culture dish) were heat shocked at 43 °C for 90 min in a 5% CO<sub>2</sub> humidified incubator in a serum-free (SF) medium and allowed to recover at 37 °C for 24 h. The extracellular medium was collected and centrifuged at 2,000×g for 15 min at 4 °C (Allegra 25R Centrifuge, Beckman Coulter, Indianapolis, IN, USA) to remove cells and debris. The supernatants were centrifuged at 10,000×g for 60 min at 4 °C, and then the supernatant was centrifuged again at 100,000×g for 60 min at 4 °C (Optima L-90 K Ultracentrifuge, Beckman Coulter) to obtain ECV pellets. The resulting pellets were resuspended in 1 mL of phosphate-buffered saline (PBS), transferred to microcentrifuge tubes (Beckman Coulter), centrifuged at 100,000×g for 60 min at 4 °C (Optima TLX Ultracentrifuge, Beckman Coulter), and resuspended in PBS. Determination of ECV concentration and ECV mean and mode size was performed for each ECV preparation by nanoparticle tracking analysis using the NanoSight NS300 instrument (Malvern Instruments, Westborough, MA, USA).

### Western blotting

Equal amounts of cell lysates or an equivalent number of ECV were solubilized in NuPAGE LDS sample buffer (Life Technologies, Carlsbad, CA, USA), and proteins were separated using NuPAGE 4–12% Bis-Tris gels (Life Technologies). Electrophoretically separated proteins were transferred onto nitrocellulose membranes (Tris transfer buffer

for 1 h at 30 V). Membranes were blocked with 5% BSA-Tris-buffered saline (TBS) for 1 h at 25 °C. Blots were probed with mouse monoclonal antibodies against HSPB1 (SPA-800 clone G3.1, Enzo Life Sciences, Farmingdale, NY, USA) or HSPB5 (SMC-159 clone 1A7.D5, StressMarq Biosciences, Victoria, Canada) in 5% BSA dissolved in TBS supplemented with 0.1% Tween 20 (TBST) at 4 °C for 16 h followed by three 15-min washes in TBST at 25 °C. Blots were then incubated with HRP-conjugated goat anti-mouse IgG antibodies (1/1000, Thermo Fisher Scientific, Fremont, CA, USA) in 5% BSA-TBST for 1 h at 25 °C. After three 15-min washes in TBST, bands were detected by chemiluminescence using SuperSignal reagents (Thermo Fisher Scientific).

### Recombinant protein purification

pET3b human HSPB1, pET16b human HSPB5 wild type, HSPB5 S19D/S45D/S59D (TM), and HSPB5 R120G were introduced in the BL21 Rosetta strain, and the proteins were expressed by incubating the cells for 4 h at 37 °C with 350 µM IPTG. The cells were lysed by sonication in TEG buffer (25 mM Tris, pH 8.0, 2 mM EDTA, 50 mM glucose) and then centrifuged at 16,000×g for 45 min at 4 °C. Supernatants first fractionated on a DEAE–Sephacose column and subsequently purified on a Source 15Q HR 16/10 column, using a NaCl gradient from 0 to 1000 mM in 2 mM EDTA, 25 mM Tris–HCl/Bis-Tris–HCl, pH 8.0.

### Liposome preparation and incorporation of proteins

Liposomes were formed by resuspending the dried lipid film (400 µg, Avanti Polar Lipids, Alabaster, AL) in 50-mM Tris Buffer, pH 7.4 (120 µl), and vortexed every 5 min for 30 s. The preparation was extruded through a 100-nm membrane filter (15 passages). Thereafter, liposomes were incubated with recombinant HSPB1 or HSPB5 and resuspended in 50-mM Tris Buffer, pH 7.4 for 30 min at 25 °C at a ratio of 400 µg lipids per 1 µg of protein or as indicated in the figure legend. After the incubation, the mixture was centrifuged at 100,000×g for 40 min at 4 °C and the pellet was washed once with sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>, pH 11.5) and centrifuged again. The final pellet, after centrifugation (proteoliposomes), was resuspended in lithium dodecyl sulfate (LDS) sample buffer and boiled for 8 min. The material was resolved by LDS-polyacrylamide gel electrophoresis (PAGE), transferred onto a nitrocellulose membrane, and the presence of HSPB1 or HSPB5 was detected by HSPB1 (SPA-800) or HSPB5 (SMC-159) as described above.

### Mass spectrometry analysis

Recombinant HSPB1 or HSPB5 (2 µg) was incubated with POPS liposomes (400 µg) in 50-mM Tris buffer, pH 7.4 for

30 min at 25 °C. Liposomes were centrifuged at 100,000×g for 40 min at 4 °C. Pellets were resuspended in Na<sub>2</sub>CO<sub>3</sub> buffer (pH 11.5) and centrifuged again. The resulting proteoliposomes were incubated with 50 µg/mL proteinase K for 30 min at 37 °C, and the liposomes were pelleted at e centrifugation and washed again. Pellets were solubilized and digested with trypsin. The resulting peptides were analyzed by HPLC coupled with tandem mass spectrometry (LC-MS/MS) using nano-spray ionization (TripleTOF 5600 hybrid mass spectrometer (AB SCIEX)). Data were analyzed using MASCOT® (Matrix Science) and Protein Pilot 4.0 (AB SCIEX) for peptide identifications.

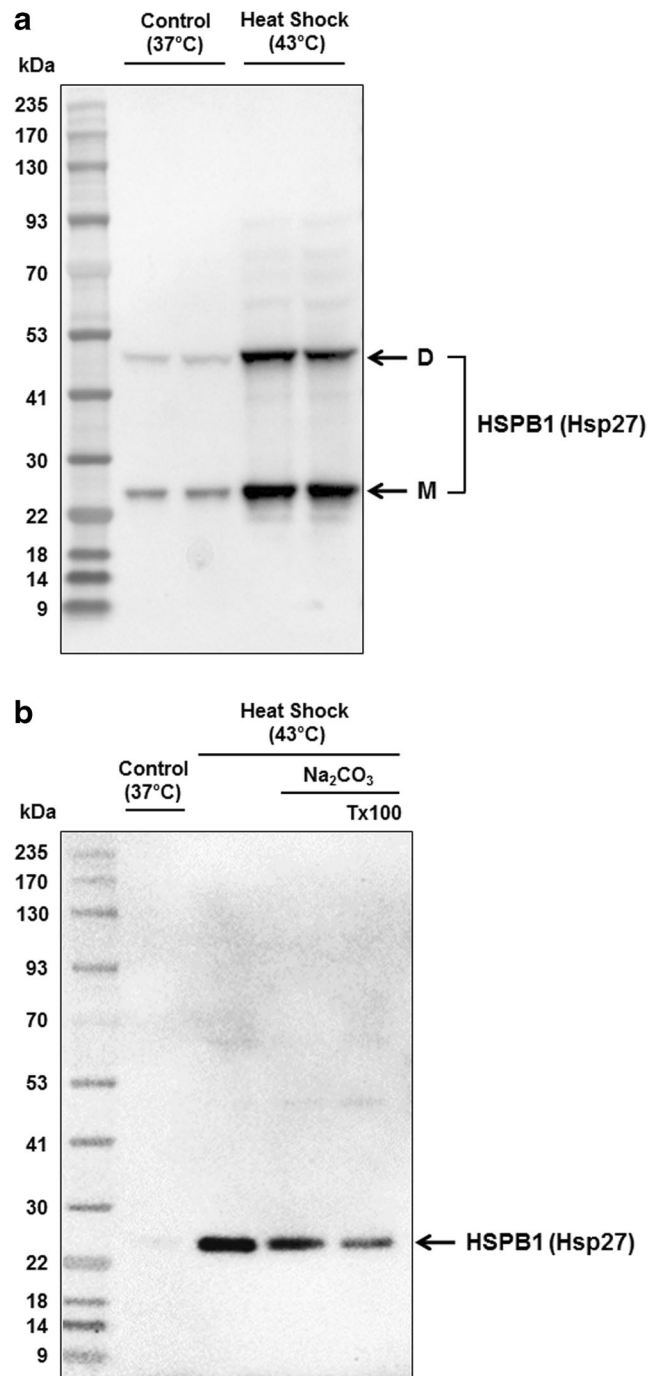
## Results

### HSPB1 is present in ECV isolated from heat shock cells

HSPB1 (Hsp27) has been detected in the extracellular space and has been proposed to be released via ECV or exosomes (Clayton et al. 2005; Nafar et al. 2016; Reddy et al. 2018; Stope et al. 2017). We evaluated whether HSPB1 was indeed present within ECV. HepG2 cells (human hepatoblastoma) express low levels of HSPB1 in normal culture conditions, but their expression is dramatically increased upon heat stress at 43 °C, with the detection of monomers and an apparent dimer (Fig. 1a); the latter likely formed by a disulphate bridge in the HSPB1 dimer interface (Almeida-Souza et al. 2010). The extracellular medium (serum-free) of HepG2 cells after heat shock (43 °C) and 24 h of recovery at 37 °C or not was collected, and ECV were isolated by differential centrifugation (Vega et al. 2008). Whereas HSPB1 was not detectable in ECV obtained from non-stressed cells (Fig. 1b), its presence was strongly detected in vesicles isolated after heat shock. However, only the monomeric form was observed (Fig. 1b). To address whether HSPB1 was intrinsically associated with ECV or just absorbed on the surface of the vesicles, isolated ECV were washed with Na<sub>2</sub>CO<sub>3</sub> (pH 11.5) that removes any proteins that are not integrated into the vesicle membrane. This treatment did not disrupt the detection of HSPB1 within the ECV preparation (Fig. 1b). Finally, ECV containing HSPB1 were incubated with the non-ionic detergent Triton X-100 and the detergent-resistant and soluble fractions were isolated. HSPB1 was observed in Triton X-100 resistant fraction (Fig. 1b), suggesting the protein exists, at least in part, within lipid raft-like domains of ECV.

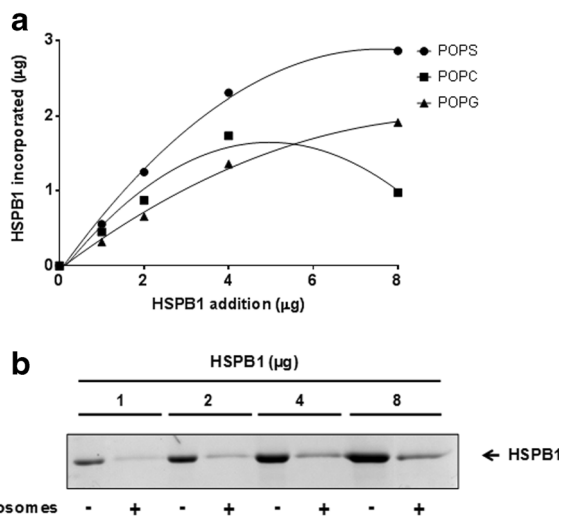
### HSPB1 associates with lipid membranes

The preceding observations suggest that HSPB1 may be inserted into the membrane of ECV. Therefore, we were wondering whether this protein could indeed interact with lipid membranes. To test this possibility, recombinant human



**Fig. 1** ECV derived from stressed cells contained HSPB1. HepG2 cells were maintained at 37 °C (control) or heat shocked (43 °C for 1.5 h) and recovered (37 °C) in serum-free medium. ECV were isolated from culture medium by high-speed centrifugation as described in [Materials and methods](#). **a** Detection of HSPB1 by western blotting in total cell lysates. **b** Detection of HSPB1 by western blotting in isolated ECV derived from control of heat-shocked cells. ECV isolated from heat-shocked cells were washed with Na<sub>2</sub>CO<sub>3</sub> (0.1 M at pH 11.5) or incubated with Triton X-100 (1% for 30 min 4 °C), centrifuged at 100,000×g for 1 h, and the pellets were analyzed for the presence of HSPB1 by western blotting. Arrows indicate the location of monomeric (m) and dimeric (d) forms of HSPB1

HSPB1 at various concentrations was incubated with liposomes made of 100% palmitoyl oleoyl phosphatidylserine (POPS), palmitoyl oleoyl phosphatidylcholine (POPC), or palmitoyl oleoyl phosphatidylglycerol (POPG), and the incorporation of the protein into the liposomes was evaluated by western blotting after pelleting the liposomes by high-speed centrifugation as described previously (Armijo et al. 2014; Lopez et al. 2016). HSPB1 avidly got incorporated into POPS liposomes in a concentration-dependent manner. The incorporation of HSPB1 into POPS liposomes displayed a saturation profile at higher amounts of the protein (Fig. 2b). HSPB1 also incorporates into POPG and POPC liposomes, but at lower levels than POPS liposomes (Fig. 2a). Representative western blotting for the incorporation of HSPB1 into POPS liposomes is presented as Fig. 2b. To further elucidate the nature of the interaction of HSPB1 with POPS liposomes, the protein was incorporated into liposomes, washed with  $\text{Na}_2\text{CO}_3$ , and treated or not with proteinase K for 30 min at 37 °C. Then, liposomes were sedimented by high-speed centrifugation, washed, and analyzed by mass spectrometry. The peptide sequences obtained from both conditions were compared (Fig. 3). The majority of HSPB1 peptide sequences detected in non-digested liposomes were also



**Fig. 2** HSPB1 got incorporated into phospholipid liposomes. Pure recombinant HSPB1 (1, 2, 4, 6, or 8 µg) was incubated with POPS, POPC, or POPG liposomes (400 µg) in 50-mM Tris buffer, pH 7.4 for 30 min at 25 °C. At the end of the incubation period, the liposomes were centrifuged at  $100,000\times g$  for 40 min at 4 °C. Pellets were resuspended in  $\text{Na}_2\text{CO}_3$  buffer (pH 11.5) and centrifuged again. Liposome pellets were then solubilized in LDS sample buffer, and liposome-incorporated proteins were separated by LDS-PAGE and analyzed by western blotting using a monoclonal antibody against HSPB1 (SPA-800, Enzo Life Sciences) and HRP-conjugated goat anti-mouse as secondary antibodies. The signal intensity of each band in the western blot was quantitated by densitometry using Bio-Rad Image Lab Software 4.1. The incorporation into the liposomes was calculated based on the signal of the corresponding signal of HSPB1 without liposomes at the respective concentration. **a** Quantification of HSPB1 incorporation into POPS, POPC, and POPG liposomes. **b** Representative western blotting for the incorporation of HSPB1 into POPS liposomes

detected after digestion of the proteoliposomes with proteinase K (bolded amino acids, Fig. 3). Only a limited number of regions were digested by the protease (marked by grey areas on Fig. 3, bottom), corresponding to domains that were exposed outside the liposome. The alpha-crystallin domain (indicated by solid underline) was highly protected except for a group of 11 amino acids suggesting that this domain is embedded into the liposome. In addition, a stretch of 19 amino acids toward the N-terminus end of the protein was not detected after Proteinase K digestion suggesting that it is exposed on the surface of the liposome (Fig. 3, bottom). Overall, this information suggests that the majority of HSPB1 is embedded within the liposomes, which could be within the membrane or in the lumen.

### HSPB5 is also present in ECV and interacts with liposomes

HSPB5 ( $\alpha\text{B}$ -crystallin) has been reported associated with ECV isolated from human retinal pigment epithelial cells (Gangalum et al. 2016). This observation was confirmed by us upon isolation of ECV from HeLa cells transfected with the HSPB5 gene (Fig. 4). Because HSPB5 is related to HSPB1, we were pondering whether HSPB5 could also interact with lipid membranes. HSPB5 was incubated in POPS, POPC, or POPG liposomes, and the incorporated protein was detected as described above. HSPB5 interacted with the three types of liposomes in a concentration-dependent manner, showing a

#### HSPB1 in POPS liposomes (without proteinase K treatment)

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1 mterrrvpfs1 lrgpswdpfr dwyphsrldf qafglprlpe
41 ewsqwlgss wpgyvrlpp aaiespavaa paysralsrq
81 lssgvseirh tadrwrvsld vnhfapdelt vktkdqvvei
121 tqkheerqde hqvisrcftr kytlppqvdp tqvssslspe
161 qtlitveapmp klatqsneit ipvtfesraq lggpeaaksd
201 etaak

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#### HSPB1 in POPS liposomes (with proteinase K treatment)

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1 mterrrvpfs1 lrgpswdpfr dwyphsrldf qafglprlpe
41 ewsqwlgss wpgyvrlpp aaiespavaa paysralsrq
81 lssgvseirh tadrwrvsld vnhfapdelt vktkdqvvei
121 tqkheerqde hqvisrcftr kytlppqvdp tqvssslspe
161 qtlitveapmp klatqsneit ipvtfesraq lggpeaaksd
201 etaak

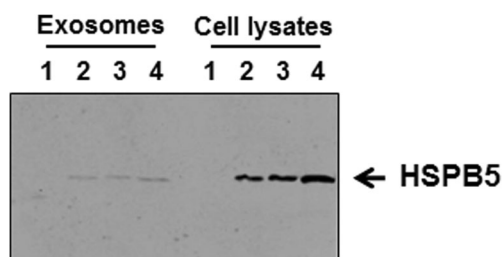
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**Fig. 3** Detection of the region of HSPB1 exposed outside POPS liposomes. Recombinant HSPB1 (2 µg) was incubated with POPS liposomes (400 µg) in 50-mM Tris buffer, pH 7.4 for 30 min at 25 °C. The liposomes were centrifuged at  $100,000\times g$  for 40 min at 4 °C. Pellets were resuspended in  $\text{Na}_2\text{CO}_3$  buffer (pH 11.5) and centrifuged again. The resulting proteoliposomes were incubated (bottom) or not (top) with Proteinase K (50 µg/mL) for 30 min at 37 °C, and the liposomes were centrifuged at  $100,000\times g$  for 40 min at 4 °C and washed again. The liposome pellet was analyzed by mass spectrometry. The peptides that were protected from Proteinase K digestion are bolded, the region that was lost after Proteinase K treatment (exposed outside the liposome) is highlighted in grey. The alpha-crystallin domain was indicated by solid underline

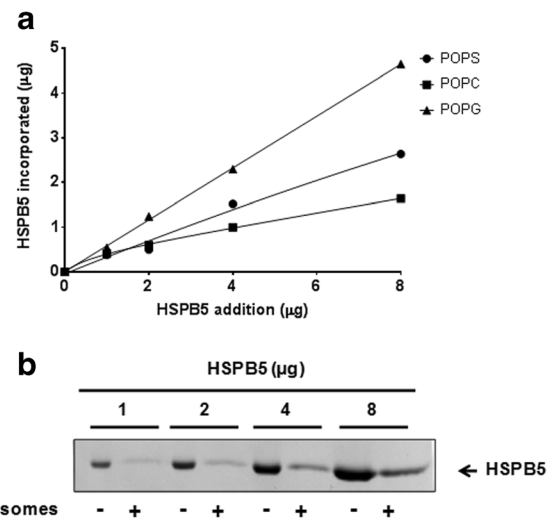
higher affinity for POPG liposomes than POPS or POPC liposomes. However, saturation was not reached in the range of concentrations used for either liposome preparation (Fig. 5a). A representative western blot for the incorporation of HSPB5 into POPS liposomes is presented in Fig. 5b. The regions in HSPB5 incorporated into POPS liposomes were also analyzed by proteinase K digestion as described above. We found that most regions were not sensitive for the protease incubation. Only two small peptides were digested by the protease treatment, particularly at the C-terminus end of the protein (see grey areas on Fig. 6), suggesting that this region is exposed outside the liposome. The alpha-crystallin domain (indicated by solid underline) was protected with the exception of a stretch of 8 amino acids. This observation suggests that HSPB5 is more embedded into the liposome than HSPB1, which is consistent with the lack of saturation in the incorporation into liposomes.

### Two mutants of HspB5 display different patterns of liposome incorporation than the wild-type protein

We investigated the presence of HSPB5 mutants within ECV and their interaction with lipid membranes. We used a triple mutant (TM) in which three serine amino acids were substituted with aspartic acids (S19D, S45D, and S59D) to mimic the potential effect of phosphorylation (see \* in Fig. 6). The second mutant has a single amino acid change in which R120 was changed to G (R120G) that has been associated with myopathy (Vicart et al. 1998). These two mutants were also detected in ECV isolated from HeLa cells transfected with these constructs (Fig. 4). First, we analyzed the interaction with POPS liposomes. In contrast with the wild type, both mutants presented a saturation pattern with liposomes made of this lipid. The initial rate of incorporation was more elevated for R120G than TM and WT (Fig. 7a). Similar saturation incorporation curves were observed for both mutants (TM and R120G) in comparison with WT after incubation with POPC or POPG



**Fig. 4** HSPB5 is present in ECV vesicles derived from transfected HeLa cells. HeLa cells were transfected with plasmid corresponding to empty vector (1), wild-type HSPB5 (2), HSPB5 triple mutant (TM) in which three serine amino acids were substituted with aspartic acids (3), or a mutant (R120G) in which a single amino acid R120 was changed to G (4). ECV were isolated from culture medium by high-speed centrifugation. The presence of HSPB5 or mutants was detected in total cell lysates or isolated ECV by western blotting. Arrow indicates the location of HSPB5



**Fig. 5** HSPB5 got incorporated into phospholipid liposomes. Pure recombinant HSPB5 (1, 2, 4, 6, or 8 µg) was incubated with POPS, POPC, or POPG liposomes (400 µg) in 50-mM Tris buffer, pH 7.4 for 30 min at 25 °C. At the end of the incubation period, the liposomes were centrifuged at 100,000×g for 40 min at 4 °C. Pellets were resuspended in Na<sub>2</sub>CO<sub>3</sub> buffer (pH 11.5) and centrifuged again. Liposome pellets were then solubilized in LDS sample buffer, and liposome-incorporated proteins were separated by LDS-PAGE and analyzed by western blotting using a monoclonal antibody against HSPB5 (SPA-800, Enzo Life Sciences) and HRP-conjugated goat anti-mouse as secondary antibodies. The signal intensity of each band in the western blot was quantitated by densitometry using Bio-Rad Image Lab Software 4.1. The incorporation into the liposomes was calculated based on the signal of the corresponding signal of HSPB5 without liposomes at the respective concentration. **a** Quantification of HSPB5 incorporation into POPS, POPC, and POPG liposomes. **b** Representative western blotting for the incorporation of HSPB5 into POPS liposomes.

#### HSPB5 in POPS liposomes (without proteinase K treatment)

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1 mdiaihhpwi hrpffpfhsp* srlfdqffge hllsdlfpt
41 stslspfylyr ppsflrapsw fdtglsemrl ekdrfsvnld
81 vkhfspelk kvvlqdviev hpkheerqde hqfiserfhr
121 kyripadvdp ltitsslssd qvltnvqprk qvsgpertip
161 itreekpavt aapkk

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#### HSPB5 in POPS liposomes (with proteinase K treatment)

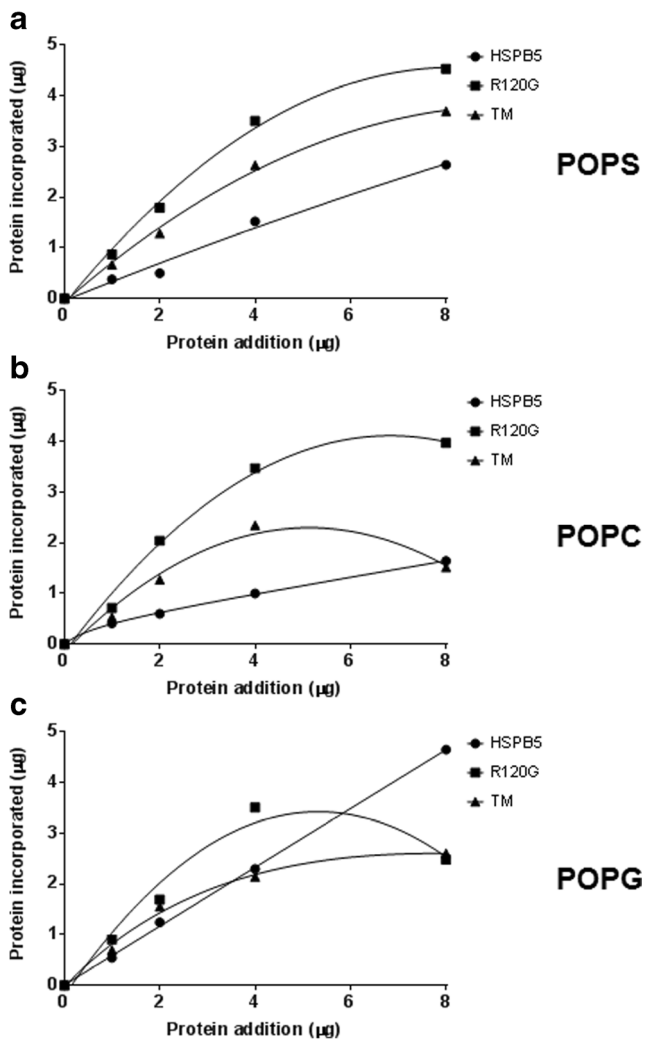
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1 mdiaihhpwi hrpffpfhsp srlfdqffge hllsdlfpt
41 stslspfylyr ppsflrapsw fdtglsemrl ekdrfsvnld
81 vkhfspelk kvvlqdviev hpkheerqde hqfiserfhr
121 kyripadvdp ltitsslssd qvltnvqprk qvsgpertip
161 itreekpavt aapkk

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**Fig. 6** Detection of the region of HSPB5 exposed outside POPS liposomes. Recombinant HSPB5 (2 µg) was incubated with POPS liposomes (400 µg) in 50-mM Tris buffer, pH 7.4 for 30 min at 25 °C. The liposomes were centrifuged at 100,000×g for 40 min at 4 °C. Pellets were resuspended in Na<sub>2</sub>CO<sub>3</sub> buffer (pH 11.5) and centrifuged again. The resulting proteoliposomes were incubated (bottom) or not (top) with Proteinase K (50 µg/mL) for 30 min at 37 °C, and the liposomes were centrifuged at 100,000×g for 40 min at 4 °C and washed again. The liposome pellet was analyzed by mass spectrometry. The peptides that were protected from Proteinase K digestion are bolded; the region that was lost after proteinase K treatment (exposed outside the liposome) is highlighted in grey. The alpha-crystallin domain was indicated by solid underline

liposomes (Fig. 7b, c). The initial rate of incorporation was also elevated in both cases for the mutant proteins as opposed to WT (Fig. 7b, c). These observations suggest that amino acid modifications within the protein altering its overall charge modify the interaction with lipid membranes.



**Fig. 7** HSPB5 or two different mutants got incorporated into phospholipid liposomes. Pure recombinant HSPB5, an HSPB5 triple mutant (TM) in which three serine amino acids were substituted with aspartic acids (S19D, S45D, and S59D), or a mutant (R120G) in which a single amino acid R120 was changed to G at various concentrations (1, 2, 4, 6, or 8 µg) was incubated with POPS (a), POPC (b) or POPG (c) liposomes (400 µg) in 50-mM Tris buffer, pH 7.4 for 30 min at 25 °C. At the end of the incubation period, the liposomes were centrifuged at 100,000×g for 40 min at 4 °C. Pellets were resuspended in Na<sub>2</sub>CO<sub>3</sub> buffer (pH 11.5) and centrifuged again. Liposome pellets were then solubilized in LDS sample buffer, and liposome-incorporated proteins were separated by LDS-PAGE and analyzed by western blotting using a monoclonal antibody against HSPB5 (SPA-800, Enzo Life Sciences) and HRP-conjugated goat anti-mouse as secondary antibodies. The signal intensity of each band in the western blot was quantitated by densitometry using Bio-Rad Image Lab Software 4.1. The incorporation into the liposomes was calculated based on the signal of the corresponding signal of the respective recombinant protein without liposomes at the various concentrations.

## Discussion

Many proteins localized within the cytosol escape this environment and are exported outside cells by an active mechanism independent of cell death. Since these proteins do not display a signal to be secreted by the ER-Golgi pathway, they are released by non-classical secretory pathway (Nickel and Seedorf 2008). These proteins within the extracellular milieu play many critical roles, particularly in signaling and the activation of target cells (De Maio 2011). A great example of proteins exported by this alternative pathway is IL-1 $\beta$  that is a key player in the inflammatory process (Eder et al. 2008). Other examples include hsp that have been detected in the extracellular medium of cells in culture as well as in many body fluids (De Maio 2011). The major challenge for a protein to translocate from the cytosol into the extracellular environment is to cross the hydrophobic barrier formed by the plasma membrane. Several mechanisms have been proposed to explain this process, including protein pores, such as ABC transporters, a lysosome-endosome pathway, exosomes, and the direct crossing of the lipid bilayer (De Maio 2011; Nickel and Seedorf 2008). In this regard, members of the Hsp70 family have been reported to interact with lipid membranes within liposomes (Armijo et al. 2014; Lopez et al. 2016) and artificial lipid bilayers (Arispe and De Maio 2000; Vega et al. 2008), reviewed by Balogi et al. 2019. In addition, these proteins have been detected on the surface of transformed cells in a membrane-anchored fashion (Multhoff and Hightower 1996). Small hsp have also been observed within mitochondrial and Golgi membranes (Whittaker et al. 2009; Gangalum and Bhat 2009; Gangalum et al. 2004). In addition, they have been detected within lens membranes (Boyle and Takemoto 1996; Cenedella and Fleschner 1992; Cobb and Petrash 2000, 2002; Friedrich and Truscott 2010), and the binding was increased by elevations of temperature (Friedrich and Truscott 2010). Membrane association has been correlated with the development of cataracts (Boyle and Takemoto 1996; Cenedella and Fleschner 1992; Cobb and Petrash 2002).

We detected the presence of HSPB1 within ECV or exosomes after heat shock (43 °C) and 24 h of recovery at 37 °C in HepG2 cells corroborating prior observations (Clayton et al. 2005; Nafar et al. 2016; Stope et al. 2017). In addition, we observed HSPB5 in ECV isolated from HeLa cells transfected with this gene, echoing other reports (Gangalum et al. 2016; Kore and Abraham 2016). Our observations suggest that HSPB1 is inserted into the ECV membrane since it was not released after washes with Na<sub>2</sub>CO<sub>3</sub> (pH 11). Moreover, the presence of HSPB1 in ECV was resistant to TX-100 solubilization suggesting that the protein is within lipid raft-like domains of these vesicles. Previous studies have also shown that HSPB5 is present within detergent-resistant membranes and released in association with exosomes (Gangalum et al. 2011, 2016). The observation that

HSPB1 and HSPB5 may be in lipid raft-like domains within ECV is not surprising since these vesicles are very rich in cholesterol that is a major component of these domains in membranes (Brown and London 2000). Moreover, Hsp70 (HSPA1) has also been reported to be present within detergent-resistant domains in ECV (Vega et al. 2008). Therefore, lipid raft regions have been proposed as one of the sites for ECV formation and release, particularly ectosomes (De Maio 2011). The presence of hsp within ECV has been linked to a signaling ability rather than a chaperone activity (De Maio 2011). Gangalum et al. (2016) have recently shown that HSPB5 could play an important role in exosome secretion, without affecting their formation. The observations that HSPB5 is located within the vesicle membrane may place the protein in direct contact with the machinery involved in exosome release, perhaps explaining the inhibition of ECV export in the absence of the protein.

The direct interaction of HSPB1 and HSPB5 with lipid membranes was investigated using liposomes followed by high-speed centrifugation and detection by western blotting as previously described (Armijo et al. 2014; Lopez et al. 2016). Both proteins displayed an affinity for various phospholipids but showed distinct specificities, suggesting that their interaction with lipid membranes may not be identical. HSPB1 reached saturation upon incorporation into liposomes, particularly POPS, at higher concentrations of the protein, whereas the incorporation of HSPB5 is still linear in the same range of concentrations. These differences may be explained by the assumption that HSPB5 is more compressed into the liposomes or perhaps driven toward the liposome lumen providing enough space to incorporate more polypeptides. A comparison of the proteomic analysis for the surface exposure of both proteins within POPS liposomes indicated that HSPB1 and HSPB5 are embedded differently into the lipid bilayer. Prior studies have shown that HSPB5 indeed interacted with lipid membranes (Borchman and Tang 1996; Ifeanyi and Takemoto 1991; Tjondro et al. 2016). The interaction of HSPB5 with lipids was not specific for the type of phospholipids (Cobb and Petrash 2002) and it was reduced by the presence of cholesterol within the membrane (Tang et al. 1998). HSPB5, as well as Hsp17, have been reported to stabilize artificial membranes mediated by interaction with the polar head group and affecting the hydrophobic region of the phospholipid (Tsvetkova et al. 2002). This report is consistent with our observations for the robust interaction of HSPB5 and HSPB1 with lipid membranes.

Two mutants of HSPB5 in which three negative charges have been incorporated into the protein (TM) or when a positive charge was deleted (R120G) showed a different behavior than wild-type HSPB5. The two mutants showed a saturation

curve for their insertion as opposed to WT, suggesting that their arrangement within the liposome is different than WT. Thus, changes in the protein charge may produce structural modifications that modulate the interaction with lipids or the packaging of the protein within the liposome. In this regard, HSPB5 has been shown to be in a non-phosphorylated form within ECV (Kore and Abraham 2016), suggesting that the excess of negative charge may interfere with packing into the vesicles. However, our observations with HSPB5 TM may not indicate that an excess of negative charge interferes with membrane interaction. These observations may indicate that both in the presence or absence of phosphate groups (negative charges) the protein can be directed to the membrane or the lumen of the vesicles. Interestingly, Kore and Abraham (2016) found that HSPB5 within exosomes was modified by O-GluNAc moieties. Indeed, HSPB5 has been reported to be modified by this cytosolic sugar entity independently of the phosphorylation stage of the protein (Roquemore et al. 1992). The saccharide moiety was added to Thr 170 of HSPB5 (Roquemore et al. 1996), which is located within the region that is exposed outside the liposome as indicated for proteinase K sensitivity.

The alpha-crystallin domain of both HSPB1 and HSPB5, which is the key feature of these proteins, has been reported to form a beta-sheet structure (Augusteyn 2004; Clark et al. 2011; Van Montfort et al. 2001). These beta-sheet structures have been proposed to form oligomeric complexes that facilitate insertion into lipid membranes (Arispe et al. 1996, 1993; Mirzabekov et al. 1996; Rojas et al. 1992). Indeed, the alpha-crystallin domain was found to be largely protected after treatment with Proteinase K after the incorporation of HSPB1 and HSPB5 into POPS liposomes, indicating that this domain is indeed inserted within the lipid bilayer. It could be that the alpha-crystallin domain may be important for the incorporation into the membrane. Indeed, the alpha-crystallin domain has been shown to be the center of oligomerization of this protein (Boelens 2014; Carra et al. 2017). Previous studies have indicated that the interaction of HSPB5 with artificial membranes is enhanced upon an increase in temperature, which has been correlated with the oligomerization of the protein (Tjondro et al. 2016). The difference in lipid binding behavior between HSPB5 and the triple mutant (TM) could be explained by differences in the oligomerization stage (Peschek et al. 2013). However, we do not know whether the oligomerization process is necessary for membrane insertion.

In summary, we showed that both HSPB1 and HSPB5 interact with lipid membranes made of a variety of phospholipids and they are also present within ECV. Therefore, it is possible that membrane insertion is the first step in their transport outside cells, which may be followed by the formation of ECV, as was previously proposed for Hsp70 (De Maio 2011).



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