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Human heat shock protein 70 (Hsp70) as a peripheral membrane protein

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ABSTRACT

While a significant fraction of heat shock protein 70 (Hsp70) is membrane associated in lysosomes, mitochondria, and the outer surface of cancer cells, the mechanisms of interaction have remained elusive, with no conclusive demonstration of a protein receptor. Hsp70 contains two Trps, W90 and W580, in its N-terminal nucleotide binding domain (NBD), and the C-terminal substrate binding domain (SBD), respectively. Our fluorescence spectroscopy study using Hsp70 and its W90F and W580F mutants, and Hsp70-ΔSBD and Hsp70-ΔNBD constructs, revealed that binding to liposomes depends on their lipid composition and involves both NBD and SBD. Association of Hsp70 with phosphatidylcholine (PC) liposomes is weak, with insertion of its Trps into the bilayer hydrocarbon region. In the presence of cardiolipin (CL), bis-monoacylglycero phosphate (BMP), or phosphatidylserine (PS) Hsp70 attaches to membranes peripherally, without penetration. Our data suggest that the organelle distribution of Hsp70 is determined by their specific lipid compositions, with Hsp70 associating with the above lipids in mitochondria, lysosomes, and the surface of cancer cells, respectively. NBD and SBD attach to lipids by extended phospholipid anchorage, with specific acidic phospholipids associating with Hsp70 in the extended conformation with acyl chains inserting into hydrophobic crevices within Hsp70, and other chains remaining in the bilayer. This anchorage is expected to cause a stringent orientation of Hsp70 on the surface. Our data further suggest that acidic phospholipids induce a transition of SBD into the molten globule state, which may be essential to allow SBD–substrate interaction also within the hydrophobic bilayer interior acyl chain region.

1. Introduction

Heat shock protein 70 (Hsp70) constitutes a highly conserved family of protein chaperones, which under physiological conditions regulate protein homeostasis and promote cell survival [1]. Some Hsps are constitutively expressed, whereas others are strictly stress-inducible [2]. The major stress-induced human Hsp70 (also referred to as Hsp72) is expressed when the cell is exposed to stress such as heat shock or UV radiation. *Escherichia coli* Hsp70 chaperone is DnaK, which is regulated by two protein modulators, DnaJ and GrpE [3]. *Saccharomyces cerevisiae* has several Hsp family members, the most studied of these being the cytosolic Ssa1p [3]. Eight different and unique Hsp70 have been reported to be present in eukaryote cells, distributed in different subcellular compartments, including cytosol, nucleus, endoplasmic reticulum, and mitochondria [2]. The main function of these ubiquitous chaperones is to bind to denatured proteins and to assist in their refolding, in order to prevent their aggregation, and to guide them to their native conformations, in a manner requiring ATP [4], thus preventing cellular damage and apoptosis induced by unfolded aggregated proteins [5]. Hsp70s consist of two domains: NBD (residues 1–386) and the C-terminal substrate binding domain (SBD, residues 386–640, [6], Fig. 1, panel A). Three distinct conformations: nucleotide free, ADP-dependent, and ATP-dependent, have been demonstrated for *E. coli* DnaK [7]. The

Abbreviations: AcrA, acrylamide; aSM, acid sphingomyelinase; a.u., arbitrary unit; BMP, bis(monoacylglycero)phosphate; Br_2PC, brominated phosphatidylcholine; 6,7Br_2-PC, 1-palmitoyl-2-(6,7-dibromo)stearoyl-*sn*-glycero-3-phosphocholine; 9,10Br_2-PC, 1-palmitoyl-2-(9,10-dibromo)stearoyl-*sn*-glycero-3-phosphocholine; 11,12Br_2-PC, 1-palmitoyl-2-(11,12-dibromo)stearoyl-*sn*-glycero-3-phosphocholine; Br_4BMP, bis[mono(9,10)-dibromostearoyl] glycerophosphate; 9,10Br_2-PS, 1-palmitoyl-2-(9,10-dibromo)stearoyl-*sn*-glycero-3-phospho-L-serine; Br_8CL, tetra(9,10-dibromo stearoyl)cardiolipin; Br_2PS, brominated phosphatidylserine; CD, circular dichroism; Chol, cholesterol; CL, cardiolipin; DnaK, *E. coli* heat shock protein 70; DTT, dithiothreitol; EDTA, ethylenediamine-N,N,N′,N′-tetraacetic acid; F, fluorescence intensity; F_0, initial fluorescence intensity; FA, fatty acid; Grp78, endoplasmic reticulum heat shock protein 70; HD, Huntington disease; HDP, host defense peptides; Hepes, 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid; Hsc70, constitutively expressed heat shock protein 70; Hsp70, Heat shock protein of ≈70kDa; Hsp70-ΔNBD, recombinant Hsp70 lacking the nucleotide binding domain; Hsp70-ΔSBD, recombinant Hsp70 lacking the substrate binding domain; Hsp70-W90F, recombinant Hsp70 with substitution W90F; Hsp70-W580F, recombinant Hsp70 with substitution W580F; KCL, potassium chloride; K_{sv}, Stern–Volmer quenching constants; L/P, lipid/protein molar ratio; LUV, large unilamellar vesicles; MES, 2-(*N*-morpholino)ethanesulfonic acid; NBD, nucleotide binding domain; NPD, Niemann–Pick disease; PEG, polyethylene glycol; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine; PS, phosphatidylserine; RFI, relative fluorescence intensity; Spm, sphingomyelin; SBD, substrate binding domain; Ssa1p, *S. cerevisiae* heat shock protein 70; tOCL, 1,1′,2,2′-tetraoleoyl cardiolipin; wtHsp70, wild type Hsp70; π, surface pressure; π_0, initial surface pressure; $\Delta\pi$, increment in surface pressure; π_c, critical packing pressure; λ, wavelength; $\Delta\lambda$, spectral center of mass

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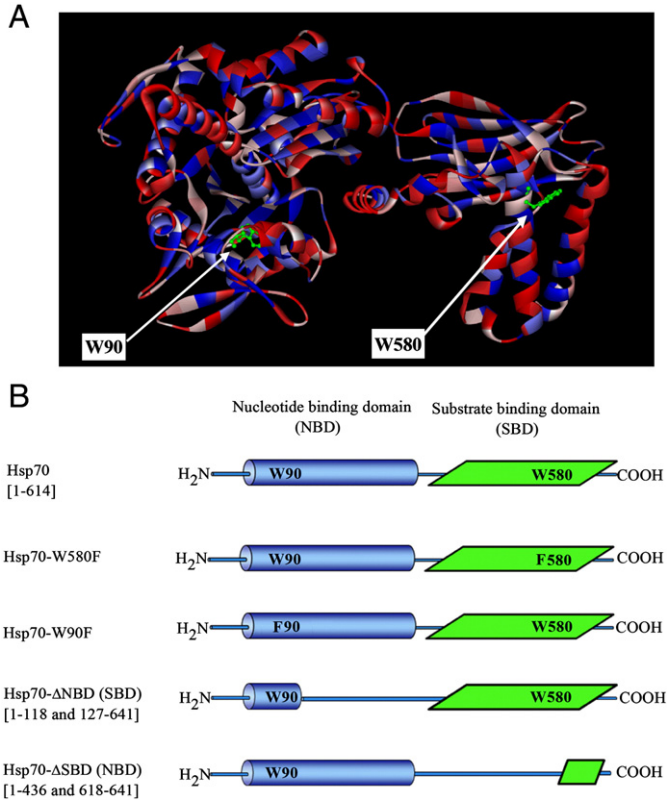

Fig. 1. Panel A: Tentative 3D ribbon structure of Hsp70 based on the crystal structures of bovine Hsc70 (PDB ID: 1YUW) and SBD of Hsp70 (PDB ID: 2P32), homology modeled by the Discovery studio. Surface coloring illustrates hydrophobicity (blue) and hydrophilicity (red). W90 and W580 are shown as ball and stick models (green). Panel B: Hsp70 constructs used in the present study.

conformations of NBD and SBD have been shown to be coupled for Hsp70 [8], DnaK [7], and Grp78 [9]. The presence of ATP accelerates the binding and release of polypeptides, but it still remains unclear whether it is the binding or hydrolysis of ATP that causes the release of peptides from SBD [8]. Formation of dimers and higher-order oligomers of Hsp70 has been suggested, with the monomeric protein representing the functionally active chaperone [10,11].

Hsp70 is additionally involved in the control of cell signaling for growth, differentiation, and apoptosis [12,13] and its overexpression is required for the growth and survival of human tumors [12–15], with elevated expression of Hsp70 correlating with poor prognosis in human breast cancer and endometrial tumors [16,17]. Hsp70 is highly expressed in the cytosol, outer surface of the plasma membrane and the membranes of the endo-lysosomal compartment in primary tumors of different origins, whereas its expression in unstressed normal cells is low and restricted to the cytosol [18–22].

Hsp70 may also provide a recognition structure for natural killer cells [23]. Multiple reports have demonstrated the association of Hsp70 family members with biomembranes in normal and tumor cells, and tumor-derived cell lines [21,23–26]. Bovine Hsc70 binds to cell surface sulfogalactolipids through its N-terminal nucleotide binding domain (NBD, [27]). Electron microscopy shows Hsp70 on the cell surface, in clathrin coated pits, and within endosome/lysosome-related vesicles [28]. There is also evidence that Hsp70 is associated with the so-called detergent resistant microdomains in the plasma membrane [29].

Direct interaction of Hsp70 with bis-monoacylglycero phosphate (BMP), an acidic phospholipid enriched in late endosomes and lysosomes [30] appears to be required for the activation of lysosomal acid sphingomyelinase (aSM), whose activity is essential for the downstream cytoprotective effect of lysosomal Hsp70. Our previous studies revealed that NBD contains a specific and pH-dependent binding site for BMP. Trp90 of NBD is required for this interaction and its mutation to Phe results in an Hsp70 with compromised BMP-binding, rendering Hsp70 unable to prevent lysosomal membrane permeabilization [30]. This interaction can also be blocked by an antibody against BMP [30].

Despite the accumulating information on the membrane association of Hsp70 and its potential significance to the functions of Hsp70, lipid–Hsp70 interactions have not been assessed in detail. Accordingly, the exact molecular mechanisms and the mode of attachment of Hsp70 to membrane bilayers remain to be elucidated. Intriguingly, Hsp70 has been demonstrated to contain two fatty acid binding sites. Membrane association of Hsp70 and its lipid-interactions demonstrated so far already suggest that Hsp70 could be a peripheral membrane protein. In this study, we exploited the intrinsic Trp fluorescence of human Hsp70 to evaluate possible lipid specificity in the membrane binding of Hsp70. Two mutants, Hsp70-W90F and Hsp70-W580F as well as NBD and SBD constructs Hsp70-ΔSBD and Hsp70-ΔNBD were additionally compared with wtHsp70 for their interactions with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), as well as cardiolipin (CL), BMP, and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS) containing PC liposomes, complementing our previous studies on BMP [30], and those by Arispe et al. [31] on PS.

The aim of the present study was to explore in more detail the interactions of Hsp70 and NBD and SBD with different lipids, providing at this stage a qualitative understanding of the interactions and how they influence the orientation and conformation of Hsp70 in membranes. Our present results demonstrate a complex array of interactions of Hsp70 with phospholipid membranes. These interactions are highly sensitive to the membrane lipid composition, with Hsp70 selectively binding to membranes containing negatively charged phospholipids such as CL, BMP, and PS. Accordingly, the distribution of Hsp70 in lysosomes, mitochondria, and on the outer surface of cancer cells, respectively, could reflect the enrichment of these lipids in the above organelles and their interactions with Hsp70. Using single Trp Hsp70 mutants W90F and W580F we showed that both NBD and SBD contribute to the attachment of Hsp70 to lipid surfaces. In NBD the phospholipid binding site involves W90, which is also involved in the cationic site responsible for the binding of ATP [32]. Our data derived from Langmuir balance and Trp fluorescence spectroscopy experiments using collisional quenching by brominated phospholipids (6,7-Br_2PC, Br_2PS, Br_4BMP, and Br_8CL), and acrylamide as well as wtHsp70, its W90F and W580F mutants, and the NBD and SBD constructs allow us to conclude that

(i) Hsp70 binds to CL, BMP, and PS containing membrane surfaces peripherally, most likely by extended phospholipid anchorage [33,34].
(ii) Both NBD and SBD appear to interact with lipids.
(iii) Our data further suggest that under these conditions SBD is likely to adopt the molten globule conformation.

2. Experimental procedures

2.1. Materials

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-(6,7-dibromo)stearoyl-*sn*-glycero-3-phosphocholine (6,7Br_2-PC), 1-palmitoyl-2-(9,10-dibromo)stearoyl-*sn*-glycero-3-phosphocholine (9,10Br_2-PC), 1-palmitoyl-2-(11,12-dibromo)stearoyl-*sn*-glycero-3-phosphocholine (11,12Br_2-PC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS), 1,1′,2,2′-tetraoleoyl cardiolipin (toCL), bis-monoacylglycero phosphate (BMP), cholesterol, N-acyl-phosphatidylethanolamine, and sphingomyelin were from Avanti Polar-Lipids Inc. (Alabaster, AL, USA). PEG 400 was from ABCR GmbH & Co.KG (Karlsruhe, Germany). Lipids were dissolved in chloroform and their concentrations were determined gravimetrically using a high precision electrobalance (Cahn, Cerritos, CA) as described