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A constitutive form of heat-shock protein 70 is located in the outer membranes of mitochondria from rat liver

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HSP73, the constitutive form of heat-shock protein 70, has been implicated in the translocation of preproteins across the mitochondrial membranes, being required for maintaining mitochondrial preproteins in an import competent conformation. Here we report that highly purified mitochondrial outer membranes contain a protein indistinguishable from HSP73 as a tightly associated peripheral component of the membrane. This membrane form of HSP73 was photolabelled with [α-^{32}P]ATP and could be released from the outer membrane with sodium carbonate, but not after incubation of the membranes with salt or with ATP. A sensitive immunoassay with an anti-HSP73 monoclonal antibody, revealed the association of HSP73 with mitochondrial outer membrane vesicles at a level similar to that of preprotein import receptors.

Mitochondrial import; Outer-membrane; Heat-shock protein; Photolabelling

1. INTRODUCTION

Most mitochondrial proteins are synthesized on free ribosomes in the cytosol as preproteins. They specifically bind to and are inserted into the mitochondrial outer membrane. It appears that preproteins must be in an unfolded state to traverse the mitochondrial membranes [1–3]. Once inside the organelle they are refolded, and various heat-shock proteins have been implicated as catalysts for this process, including HSP60 (MIF4) and mtHSP75 (SSC1), which have been shown to refold imported preproteins in the matrix of *S. cerevisiae* and *N. crassa* mitochondria [4–7]. Less is known about the events leading to interaction of the unfolded preprotein with the outer mitochondrial membrane. Cytosolic HSP73 is known to bind partly folded proteins during synthesis on free ribosomes [8]. A role has been predicted for cytosolic HSP73 in mitochondrial preprotein import in vitro [9,10] and in vivo [11]. The presence of soluble HSP73 in the in vitro import assay prolongs the loosely-folded, import-competent state of mitochondrial preproteins [10]. However, contrary to these genetic and biochemical data, the efficient import of a purified preprotein into isolated mitochondria suggests that soluble HSP73 is not required [12]. Recently the matrix-localized mtHSP75 has been found to be intimately associated with the import machinery of the mitochondrial inner membrane [13] and has been predicted to be involved in driving the translocation of preproteins across the inner membrane and into the matrix [7,14]. Recent evidence that preproteins are translocated across the two mitochondrial membranes by the sequential action of independent translocation complexes localized to each of the membranes [15,16] have led us to investigate how mitochondrial preproteins are driven through the translocation machinery of the outer membrane. Here we report that an isoform of HSP70, indistinguishable from HSP73, is tightly and specifically associated with the mitochondrial outer membrane.

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Abbreviations: HSP60, heat-shock protein of 60 kDa; HSP70, heat shock protein of 70 kDa; the HSP70 isoforms referred to in the text are: HSP73, constitutive form; HSP72, inducible form; mtHSP75, mitochondrial form; GRP78, endoplasmic reticulum form; ELISA, enzyme-linked immunosorbent assay.

2. MATERIALS AND METHODS

2.1. *Isolation of mitochondria and outer membrane vesicles*

Mitochondria were prepared from rat liver [17] and further purified on discontinuous sucrose gradients [18] by collecting the band of material at the interface of the 1.3 M and 1.6 M sucrose layers. This was diluted with four volumes of 10 mM HEPES (pH 7.6), 1 mM EDTA and 0.5 mM phenylmethylsulfonylfluoride and mitochondria recovered by centrifugation at 6,000 × *g* for 10 min. Outer membrane vesicles were prepared from these mitochondria by the method of Söllner et al. [19]. The vesicles were resuspended in 10 mM HEPES, 1 mM EDTA (pH 7.6) at a protein concentration of 2 mg/ml. Between 1–2 mg of purified outer membrane vesicles were prepared from 60 g liver.

2.2. *Purification of HSP73*

Cytosolic HSP73 was purified by ATP-Agarose affinity chromatography as described by Welch and Feramisco [20]. A monoclonal antibody, N6 F3–5 which recognizes both constitutively-expressed HSP73

and stress-inducible HSP72 was raised in mice against purified Hela HSP73/72 [21] and was kindly donated by Dr. W. Welch. Antibodies recognizing mtHSP75 (MA3-006) and GRP78 (MA3-007) were from Affinity Bioreagents.

2.3. *Resolution of mitochondrial outer membrane proteins by 2D-gel electrophoresis and immunoblotting*

An aliquot of the outer mitochondrial membrane preparation containing 75 μg of protein was dried down and resuspended in 60 μl isoelectric focussing sample buffer [22]. To this was added an aliquot of sample from RIF-TR_5 cells that had been incubated for 24 h with [^{35}S]methionine [23]. The sample was subjected to isoelectric focussing in a tube gel followed by separation based on molecular weight on a 10% slab gel as described by Duncan and Hershey [22]. The proteins were electrophoretically transferred to nitrocellulose and probed with the N6 antibody. N6 bound to the filter was detected with alkaline phosphatase-anti mouse IgG secondary antibody. After photography, the filter was exposed to Kodak XAR-2 film at −70°C.

2.4. *Photolabelling of outer membrane proteins*

Outer membrane vesicles were photolabelled with 50 μCi [α-^{32}P]ATP (sp.act. 3000 Ci/mmol) by placing 50 μl of outer membrane vesicles or cytosolic fractions (0.1 μg protein/μl) in individual wells of a 96-well tissue culture plate (Costar) in medium containing 20 mM HEPES (pH 7.6), 0.5 mM EDTA and 5 mM $MgCl_2$. Labelled nucleotide was added immediately before irradiation with UV light which was performed by inverting a transilluminator (UVP) over the plate placed in an ice water bath. UV exposure was for 15 min, after which SDS-sample buffer was added and samples analysed by 10% SDS-PAGE on 10% gels. Quantitation of ATP-binding proteins was achieved by radioscanning the gels using a Phosphorimager (Molecular Dynamics).

2.5. *Extraction of outer membrane vesicles*

Extraction of the purified membrane vesicles with salt and alkali buffers was carried out at 4°C. After photolabelling of vesicles with [α-^{32}P]ATP, the vesicles were isolated by centrifugation in a Beckman TL100.2 rotor at 55,000 rpm for 30 min. The pellet was resuspended in 50 μl of appropriate buffer with intermittent vortexing over 15 min. Membranes were extracted with 10 mM HEPES, 1 mM EDTA (pH 7.6) (low salt); 10 mM HEPES, 1 mM EDTA, 250 mM NaCl (high salt); 10 mM HEPES, 5 mM $MgCl_2$, 1 mM ATP or 100 mM Na_2CO_3. The extracted membrane vesicles were separated as described above and the vesicle pellet and supernatant fractions were analysed by SDS-PAGE on 10% gels.

2.6. *Measurement of outer membrane HSP73 levels using a quantitative ELISA*

HSP73 purified from rat liver cytosol was diluted in 77 mM Na_2CO_3–115 mM $NaHCO_3$ (pH 9.5). Labelled mitochondrial outer membrane vesicles were extracted with 100 mM Na_2CO_3 as described above and extracts buffered to pH 9.5 by the addition of 30 μl of 500 mM $NaHCO_3$. Samples (100 μl) were added to a 96-well polystyrene immunoassay plate (Flow laboratories) and assayed for levels of HSP73 using a quantitative ELISA [24]. The N6 antibody was used to detect HSP73 and a standard curve using 0 to 80 ng of purified rat liver HSP73, whose concentration was determined by amino acid analysis, was prepared for quantitation.

3. RESULTS AND DISCUSSION

3.1. *2-D Gel electrophoresis and immunoblotting of outer membrane proteins*

The N6 monoclonal antibody employed in this study recognizes both HSP73 and HSP72 on Western blots, but not mtHSP75, nor the endoplasmic reticulum localized GRP78 [23]. Preliminary results in this laboratory suggested that the N6 antibody recognized a protein of ≈ 75 kDa in highly purified mitochondrial outer membranes after SDS-PAGE. In order to characterize this outer membrane protein, mitochondrial outer membrane vesicles were mixed with [^{35}S]methionine-labelled RIF-TR_5 cells [23] and subjected to 2-D gel electrophoresis. The proteins were then transferred to nitrocellulose. A radiograph from the Western transfer of the mitochondrial outer membrane sample spiked with ^{35}S-labelled RIF-TR_5 cell proteins is shown in Fig. 1a. The positions of HSP73, HSP72, HSP60 and actin as judged from the labelling pattern, are marked. From the immunostained membrane (Fig. 1b), three spots were observed, the major one co-migrating exactly with HSP73. This distinguishes the HSP70 protein associated with the mitochondrial outer membrane from the heat-inducible (HSP72), mitchondrial matrix-located (mtHSP75) and microsomal (GRP78) isoforms of HSP70. The more basic HSP70, of similar molecular size to HSP73, did not correspond in mobility to any of the previously identified HSP70 isoforms. The amount of this protein detected was at the sensitivity limit of our immunoblotting assay, precluding further direct analysis of this novel form of HSP70. The ^{35}S-labelled RIF-TR_5 protein standards were added at a level too low for immunodetection, confirming that the immunoreactive HSP70 observed in the mixed sample is a component of the mitochondrial outer membrane vesicle preparation.

3.2. *Photolabelling of mitochondrial outer membrane proteins*

In order to investigate whether the association between HSP73 and the mitochondrial outer membrane was specific and not merely a result of cytosolic contamination, mitochondrial outer membrane proteins were photolabelled with [α-^{32}P]ATP, based on the known ability of HSP73 to bind ATP [11]. The purified HSP73 of rat liver has an apparent molecular weight of 75 kDa as determined by SDS-PAGE (Fig. 2, lane 2). Photolabelling of a cytosolic extract of rat liver results in proteins with molecular weights in the 20–50 kDa range becoming labelled with [α-^{32}P]ATP in addition to the 75 kDa ATP-binding protein corresponding to HSP73 (Fig. 2, lane 4). Purified HSP73 (Fig. 2, lane 2) and HSP73 photolabelled in the cytosolic extract (lane 4) co-migrates with the 75 kDa ATP-binding protein in outer membrane vesicles (lane 6). The identity of the photolabelled species was confirmed as being HSP73 since it could be extracted with Na_2CO_3 (see below) and the extracted material could be quantitated with a monoclonal antibody specific for HSP73. The complete absence of the smaller cytosolic ATP-binding proteins in the vesicle preparation (Fig. 2, lane 6) is a strong indication that the interaction between the photolabelled HSP73 and the mitochondrial outer membrane is not due to a contamination of this fraction by cytosol.

HSP73 has also been implicated in the translocation

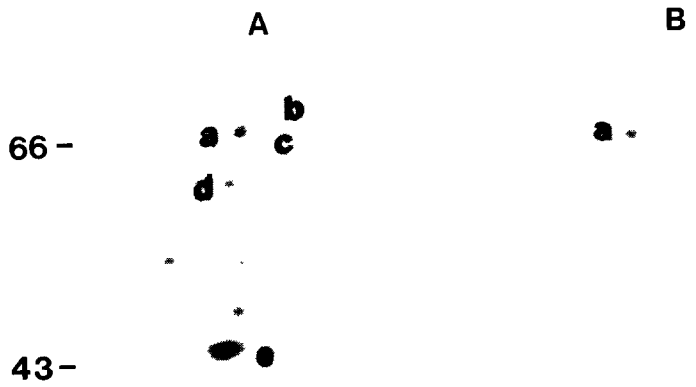

Fig. 1. Two-dimensional gel analysis of a sample of outer mitochondrial membrane proteins mixed with [^{35}S]methionine labelled whole cell proteins from RIF-TR$_5$ cells. The proteins were first separated on an isoelectric focussing gel with a pH range from 5 to 7.5, followed by separation based on molecular weight. Fig. 1a. Autoradiograph of the membrane after Western transfer showing the radioactive proteins from the RIF-TR$_5$ cells. The marked proteins are HSP73 (a), mtHSP75 (b), HSP72 (c), HSP60 (d) and actin (e). The bars on the lefthand side show the molecular mass (in kDa) of standard proteins run on the gel. Fig. 1b. Proteins from the outer membrane vesicles immunostained using the N6 antibody. The major spot (a) comigrates with HSP73.

of proteins into the endoplasmic reticulum [9], however it is unlikely that the HSP73 photolabelled in these mitochondrial membrane vesicles represents any microsomal contamination. We have previously demonstrated, with an [α-^{32}P]GTP photolabelling assay, that the mitochondrial membrane vesicles are not contaminated with microsomes to any detectable level [25]. In addition, immunoblot analysis of the mitochondrial outer membrane vesicles with antibodies to GRP78, the endoplasmic reticulum-located isoform of HSP70, reveal no detectable cross-contamination of these membrane fractions (data not shown).

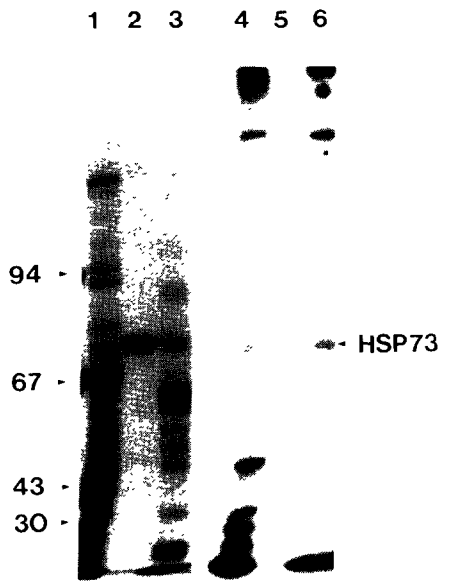

Fig. 2. The 75 kDa protein photolabelled with [α-^{32}P]ATP comigrates with HSP73 during SDS-PAGE. Protein from rat liver cytosol (lanes 1 and 4), or mitochondrial outer membranes (lanes 3 and 6) were labelled with [α-^{32}P]ATP for 15 min as described in section 2. The samples were separated on a 7% gel and the stained gel (lanes 1–3) dried before autoradiography (lanes 4–6) for 16 h. A sample of HSP73, purified from rat liver cytosol was run in lanes 2 and 5 as a marker (arrow). The size of the molecular mass markers is shown at the left.

In order to investigate the nature of the association between HSP73 and the mitochondrial outer membrane, outer membrane vesicles were UV-irradiated in the presence of [α-^{32}P]ATP. Proteins of subunit molecular mass 75 kDa and 80 kDa were labelled intensely and reproducibly (Fig. 3, lane 1). Some labelling of other proteins in the vesicle preparation was observed only after prolonged autoradiography (compare Fig. 2 and Fig. 3). The 80 kDa ATP-binding protein is an integral membrane protein since it could not be removed from the vesicles by treatment with low salt (Fig. 3, lanes 2 and 3), high salt (lanes 4 and 5), 1 mM ATP (lanes 6 and 7) or alkali (lanes 8 and 9). The 75 kDa ATP-binding protein corresponding to HSP73, however, was largely solubilized from the membranes after sodium carbonate treatment (lanes 8 and 9), but not after any of the other treatments. The tendency of HSP73 to bind loosely-folded polypeptides [8] raises the possibility that the association of HSP73 is via denatured targets on the outer membrane. However the failure of ATP to release any HSP73 from the membrane strongly argues against this explanation. In addition, we have consistently failed to observe any association between soluble, radio-labelled HSP73 and isolated mitochondria or mitochondrial outer membrane vesicles, suggesting that such artefactual targets for HSP73 do not exist on the surface of the organelles. Instead, we believe a defined number of binding sites exist in the mitochondrial outer membrane, and that these remain occupied by a population of HSP73. Our conclusion is that a population of HSP73 exists as a tightly-associated, peripheral protein of the mitochondrial outer membrane.

What might be the role of a population of HSP73

Fig. 3. Extraction of HSP73 from the mitochondrial outer membrane. Mitochondrial outer membrane vesicles were irradiated for 15 min in the presence of [α-^{32}P]ATP as described in section 2. Labelled vesicle proteins were electrophoresed on a 12.5% SDS-polyacrylamide gel and after staining with Coomassie blue, the gel was dried for autoradiography for 72 h. The position of labelled proteins of 80 kDa and 75 kDa (HSP73), are based on electrophoresis of markers. Photolabelled vesicles (lane 1) were reisolated by centrifugation and resuspended by vortexing in 10 mM HEPES, pH 7.4, 1 mM EDTA (lanes 2 and 3), 10 mM HEPES, 1 mM EDTA, 250 mM NaCl (lanes 4 and 5), 10 mM HEPES, 5 mM $MgCl_2$, 1 mM ATP (lanes 6 and 7) or 100 mM Na_2CO_3 (lanes 8 and 9). Pellet fractions are in lanes 2, 4, 6 and 8 and supernatant fractions in lanes 3, 5, 7 and 9.

bound so tightly to the mitochondrial surface? Quantitation of HSP73 in the outer membrane fraction by ELISA showed that there is 12 ng of alkali extractable HSP73/μg membrane protein. It has been estimated that in *Neurospora*, the mitochondrial preprotein receptors represent a similar proportion of the mitochondrial outer membrane [26]. Given this similar abundance, and the ability of HSP73 to stabilize loosely-folded preproteins [10], we propose that the outer membrane form of HSP73 could interact specifically with a protein component of the translocation complex where preprotein import occurs [27,28] thereby driving the transfer of preproteins from the cytosol into the translocation apparatus of the outer membrane. The release of polypeptides from HSP73 requires ATP [29] and at least one event requiring ATP does take place on the mitochondrial surface during the import of some preproteins [30,31]. ATP appears also to be required in the assembly of monoamine oxidase into the mitochondrial outer membrane [32]. The membrane form of HSP73 may be required to assist in the assembly of proteins located in the mitochondrial outer membrane. In the case of preproteins translocated across the membrane, the presence of HSP73 as a peripheral component of the membrane provides a means to transfer a loosely-folded polypeptide directly into the translocation apparatus. It seems that mtHSP75 receives translocated preproteins directly from the translocation apparatus [13] prior to the completion of folding, catalyzed by chaperonin60 and chaperonin10 [7,33]. It has recently been shown that the process of protein folding in *E. coli* requires the sequential action of a series of molecular chaperones, such that the polypeptide is released from one chaperone directly onto the next chaperone in the series [34]. Our finding that HSP73 is tightly associated with the mitochondrial outer membrane, and the demonstrated association of mtHSP75 with the inner membrane, provides a corridor to connect this series of molecular chaperones in distinct cellular compartments for the process of folding.

The finding of HSP73 in the mitochondrial outer membrane is in accord with the demonstrated presence of forms of HSP70 in the chloroplast envelope [35,36], the *E. coli* cytoplasmic membrane [37] and the recent isolation of a cDNA clone encoding SCE70, an isoform of HSP70 which is targeted to the outside of the chloroplast envelope by a non-cleavable transit sequence [38]. This chloroplast isoform is more closely related to the cytosolic forms of HSP70 than forms found inside organelles and as suggested by Ko et al. [38] opens up the possibility that members of the HSP70 family reside in all membranes across which loosely-folded polypeptides are translocated. A component of the translocation apparatus in the membrane of the endoplasmic reticulum has been shown to contain a domain suitable to serve as a HSP70-binding site [39]. Studies with yeast and *Neurospora* have identified a number of components of the mitochondrial outer membrane that interact with various preproteins during translocation across the mitochondrial membranes [27,28] and genetic analysis has revealed that the yeast homologues of HSP73 have an essential role to play in the process of preprotein translocation [9]. Determining precisely the integral proteins of the mitochondrial outer membrane which might serve as a binding site for HSP73 awaits the purification and reconstitution of such membrane components into liposomes and the use of chemical crosslinking methods to determine the overall architecture of the translocation complexes.

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