PBP74, a New Member of the Mammalian 70-kDa Heat Shock Protein Family, Is a Mitochondrial Protein

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The cloning of a cDNA encoding a new member of the highly conserved mammalian 70-kDa heat shock protein (hsp 70) family termed PBP74 was recently reported. Critical to an understanding of the function of this new hsp 70 is delineating its subcellular localization. Here we use a variety of immunological and biochemical approaches both *in vitro* and *in vivo* to demonstrate that PBP74 is imported into and resides in mitochondria. By confocal immunofluorescence microscopy PBP74 is detected in mitochondria, colocalizing with the mitochondrial 60-kDa heat shock protein. To address the inherent problem of serological cross-reactivity among the hsp70 family members, an influenza virus hemagglutinin epitope tag was introduced into the PBP74 cDNA. The epitope-tagged PBP74 protein transiently expressed in L cells localized to mitochondria. Moreover, deletion of the N-terminal 46-amino acid presequence results in a cytosolic localization of the epitope-tagged protein. Cell fractionation studies demonstrated PBP74 in purified mitochondria in a protease-protected location. After coupled transcription-translation the precursor of PBP74 is imported into isolated yeast mitochondria, where it becomes processed to the mature protein. According to a subfractionation of the mitochondria, the imported protein was found to be localized in the matrix space. Import *in vitro* is time- and temperature-dependent, requires matrix ATP, and is abolished upon depletion of the membrane potential across the mitochondrial inner membrane. Similarly, in mammalian cells PBP74 is synthesized as a pre-protein that requires membrane potential-dependent import into mitochondria for its maturation. Taken together, our data demonstrate that PBP74 is a mammalian mitochondrial hsp70.

INTRODUCTION

The 70-kDa heat shock protein (hsp70) family is a highly conserved, highly abundant family of proteins that function as molecular chaperones (reviewed in Gething and Sambrook, 1992). As the name implies, the hsp70 family was first identified because of the dramatic and specific transcriptional induction of one member, namely hsp70, after stress in the form of elevated temperatures or heat shock (reviewed in Lindquist and Craig, 1988). However, it is now clear that the majority of the members of this family are expressed constitutively in the absence of stress and are required for cell viability under normal growth conditions. Hsp70s are involved in all aspects of protein

folding and oligomerization as well as in the intracellular transport of folding intermediates to their appropriate destinations. Each is composed of a highly conserved, well characterized N-terminal ATP-binding domain and a less conserved C-terminal domain that confers the ability to bind peptides and denatured or unfolded proteins. Members of the hsp70 family have been identified that reside primarily in the cytosol (hsc70, hsp70), endoplasmic reticulum (ER) (BiP/grp78), and mitochondria (grp75) (reviewed in Gething and Sambrook, 1992). In mammals the genes encoding the cytosolic hsc70, hsp70, and ER BiP/grp78 have been isolated; however, the gene encoding the mammalian mitochondrial hsp70, grp75, has not been reported.

Recently, the cDNA for a new member of the mammalian hsp70 family was reported independently by three laboratories (Domanico *et al.*, 1993; Michikawa *et al.*, 1993b; Wadhwa *et al.*, 1993a). At present there is considerable confusion concerning the identity of this new hsp70 member and its function. We isolated both a mouse and a human cDNA, encoding a 74-kDa peptide-binding protein (PBP74), by oligonucleotide screening of cDNA libraries using information from ~60 residues of amino acid sequence derived from the purified protein. PBP74 was one of several peptide-binding proteins isolated from spleen cells by peptide-affinity chromatography in our studies aimed at characterizing potential molecular chaperones playing a role in the immune system in antigen processing. Independently, Michikawa *et al.* (1993b) isolated a mouse cDNA encoding C3H strain-specific antigen (CSA), an antigenic protein specific for the C3H mouse strain. The PBP74 cDNA was also reported by Wadhwa *et al.* (1993a) as encoding a 66-kDa antiproliferative protein, termed mortalin, associated with mortal phenotypes. In addition to the ambiguity concerning the function of PBP74, it is unclear where PBP74 resides within the cell. In a preliminary examination by immunofluorescence, we observed a predominant vesicular staining in mouse cells using peptide-affinity purified rabbit antibodies directed against PBP74, together with a weak staining of mitochondria (Domanico *et al.*, 1993). Using a CSA-specific monoclonal antibody (mAb), Michikawa *et al.* (1993b) showed an exclusive mitochondrial staining pattern in mouse cells. Based on the structural organization of the gene encoding CSA, these authors further speculated that the protein might have the same localization as cytochrome c_1 , in the intermembrane space of mitochondria (Michikawa *et al.*, 1993a). Wadhwa and colleagues (Wadhwa *et al.*, 1993a,b; Kaul *et al.*, 1993), using a rabbit antiserum raised to the purified mortalin protein, showed by immunofluorescence microscopy and cell fractionation a cytosolic and a perinuclear localization.

Having determined the N-terminal amino acid sequence of the mature PBP74 protein, we were able to deduce that the cDNA encodes a 46-amino acid presequence not present in the mature protein (Domanico *et al.*, 1993). The presequence displays structural similarities to mitochondrial-targeting sequence motifs that may form amphipathic helices but does not conform to these motifs completely (von Heijne, 1986; Domanico *et al.*, 1993). Thus, although the presequence could not be taken as definitive evidence that PBP74 was present in the mitochondria, it suggested that PBP74 might encode a mitochondrial hsp70. Because the subcellular localization of PBP74 is essential for an understanding of its function, we addressed this problem in some detail. To circumvent the inherent ambiguity of the previous serological analyses, we used a number of independent biochemical and immunological criteria both *in vivo* and

in vitro to demonstrate the exclusive localization of PBP74 to mitochondria, where it resides in the mitochondrial matrix. Thus, PBP74 cDNA encodes the mammalian homologue of the well-characterized *Saccharomyces cerevisiae* mitochondrial hsp70, Ssc1p (Craig *et al.*, 1989; Kang *et al.*, 1990). Presumably, PBP74 is identical to the mitochondrial hsp70 protein, grp75, that was identified by biochemical means (Mizzen *et al.*, 1989).

MATERIALS AND METHODS

Cell Lines

DAP.3 is a mouse fibroblast L-cell line kindly provided by Dr. R. Germain (National Institutes of Health, Bethesda, MD). CH27 is a mouse B cell lymphoma (Haughton *et al.*, 1986) characterized and kindly provided by Dr. Geoff Haughton (University North Carolina, Chapel Hill). Cells were cultured in complete Delbucco's modified Eagle's medium (DMEM) (Jelachich *et al.*, 1984) containing 10% fetal calf serum (10% CM) at 37°C and under a 5% CO₂ atmosphere. All cell lines are routinely monitored for mycoplasma and are free of this contamination.

Antibodies and Peptides

mAb 12CA5 ascites, specific for an epitope of the influenza hemagglutinin (HA) protein (YPYDVPDYA) (Wilson *et al.*, 1984), were purchased (Berkeley Antibody, Richmond, CA). A synthetic peptide of the HA epitope was synthesized, purified, and kindly provided by Dr. Pravin Kaumaya (Ohio State University, Columbus, OH). The mitochondrial 60-kDa heat shock protein (mt hsp60) specific mAb, II-13, was kindly provided by Dr. R. Gupta (McMaster University, Hamilton, Ontario, Canada). An antiserum was generated in a rabbit against the predicted C-terminus of PBP74, residues 615–633. A rabbit was immunized with a synthetic hybrid peptide consisting of a tetanus toxoid (TT) peptide, residues 582–599 (Ho *et al.*, 1990) at the N-terminus, joined by a four-residue β -turn (GPSL) to a peptide of PBP74 residues 615–633 at the C-terminus (TT582-599-PBP74-615-633). The peptide was synthesized, purified, and kindly provided by Dr. Pravin Kaumaya (Ohio State University). The TT peptide provides a potent T-cell antigenic determinate, and recent studies have shown that such hybrid peptides are strong immunogens (Kaumaya *et al.*, 1990). The rabbit was injected subcutaneously with 0.5 mg of TT582-599-PBP74-CSA-615-633 in 500 μ l of water emulsified with 500 μ l Titer-Max (CytRx, Atlanta, GA) and was bled at two to three weekly intervals. Antibodies specific for PBP74 615-633 were affinity purified on a PBP74 615-633-Sepharose column as described (Domanico *et al.*, 1993). An antiserum was also generated in a rabbit to a hybrid peptide consisting of the N-terminal peptide of the mature PBP74, residues 1–16, connected to a peptide of TT residues 582–599 at the carboxyl terminal via GPSL as detailed elsewhere (Domanico *et al.*, 1993).

HA-Epitope Tagging of PBP74 cDNA

The PBP74 cDNA was engineered to encode a nine-amino acid influenza HA antigenic epitope using polymerase chain reaction (PCR). Two separate reactions were carried out. In one reaction a PBP74 cDNA-containing plasmid pG7z-PBP1.8 (0.1 μ g) (Domanico *et al.*, 1993) was amplified 20 cycles with *Taq* DNA polymerase (Promega, Madison, WI) using primers BPBHATG4 (AGTCTGG-GACGTCGTATGGGTATTCCTTCAGATGCCATCTTT) and BPPIP12 (TGGAATTCCCCAGCC) to generate a product containing an *Eco*RI site (bold-BPIP12) internal to PBP74 (5' end) and an *Aat* II site (bold-BPBHATG4) within the engineered HA-encoding sequence (underlined-BPBHATG4) (3' end). In the second reaction the same template (0.1 μ g) was amplified 20 cycles using primers

PBPATG3 (ATACGACGTCCTCCAGACTACGCTGGTCTGGAAGTTCTGGCACTG) and PGEMSP6 (GGTGACACTATAGAA-TACTCAAG) to generate a product containing an *Aat* II site (bold-PBPATG3) within the engineered HA-encoding sequence (underlined-PBPATG3) (5' end) and a *Bam*HI site (3' end) as part of the amplified multiple cloning site. The resulting products were each digested with *Aat* II (Promega) followed by a digest with *Eco*RI (Promega) and *Bam*HI (Promega), respectively. Both digested products were subcloned together in a three piece-ligation reaction into *Eco*RI- and *Bam*HI-digested pBluescript SK (Stratagene, La Jolla, CA). One clone (pB-3'HA_{tg}) was analyzed by double-stranded DNA sequencing as previously described (Domanico *et al.*, 1993) to insure fidelity to the cDNA sequence and the designed HA tag. An *Eco*RI fragment of pG7z-PBP1.8 was subcloned into pB-3'HA_{tg} at the *Eco*RI site to generate a full-length HA-tagged cDNA, termed pB-PBPHA. The pB-PBPHA internal *Eco*RI ligation site was checked by double-stranded DNA sequencing. pB-PBPHA or pG7z-PBP1.8 plasmid DNA (1 μ g) were transcribed and translated in vitro in the coupled reticulocyte lysate system (Promega) with T7 RNA polymerase and 40 μ Ci ³⁵S-Met (Amersham, Arlington Heights, IL). For one reaction, no DNA was added to the reticulocyte lysate as a control. Samples were subjected to immunoprecipitation as described (Domanico *et al.*, 1993) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

A portion of the PBP74 cDNA lacking the 5' untranslated region (utr) was constructed by PCR. The 5' utr was removed, except the Kozak sequence including the initiating ATG (Kozak, 1991), and a *Xba* I restriction site (bold-PBPIP13) was engineered for subcloning. An internal *Pst* I restriction site was used in design of the second primer (bold-hPBPIP1) to aid in subcloning. PCR was performed on pG7z-PBP1.8 (0.1 μ g) with *Taq* DNA polymerase and primers hPBPIP1 (ATTTCTGCAGTCTCTT) and PBPIP13 (GCTCTAGAGCCACCATGATAAGCG) for 30 cycles. The PCR products were digested with *Xba* I (GIBCO-BRL, Grand Island, NY) and *Pst* I (Promega) and subcloned into pBKCMV (Stratagene). One clone (pBKCMV-5'utr(-)) was double-stranded DNA sequenced to insure fidelity to the cDNA sequence. pBKCMV-5'utr(-) was digested with *Kpn* I (Promega) and *Pst* I and subcloned into pB-PBPHA, replacing the 5' utr-containing 5' end of PBP74 cDNA. The resulting plasmid was termed pB-PBPHA.1.

A PBP74 cDNA lacking both the 5' utr and the presequence-encoding region was constructed by PCR. The presequence was removed using a primer designed to add a consensus Kozak sequence including an initiating ATG codon (Kozak, 1991) (underlined-PBPIP14) for the first amino acid (Ala) of the mature protein in addition to a *Eco*RI site (bold-PBPIP14). PCR was performed on pG7z-PBP1.8 (0.1 μ g) with *Taq* DNA polymerase and primers hPBPIP1 and PBPIP14 (CGAATTCGCCACCATGGCATCAGAAGCAATCAAG) for 30 cycles. The PCR products were digested with *Eco*RI and *Pst* I, gel purified, and subcloned into pBluescript SK. A clone (pB-preseq(-)) was identified and sequenced. A *Pst* I and *Bam* HI digest fragment of pB-PBPHA was subcloned into pB-preseq(-) at *Pst* I. The resulting vector was termed pB-PBPHA-L.

For transfection experiments the HA-tagged cDNAs with and without the presequence-encoding region were subcloned from pB-PBPHA.1 and pB-PBPHA-L, respectively, into the eukaryotic expression vector pBKCMV. pB-PBPHA.1 was digested with *Xba* I and subcloned into pBKCMV. The resulting clone, termed PBPHA, was checked by restriction mapping. pB-PBPHA-L was digested with *Xba* I and *Sal* I (Promega) and subcloned into pBKCMV. The resulting plasmid was termed PBPHA Δ pre. Insertion was confirmed by restriction mapping. All plasmid DNAs were prepared by a modified alkaline lysis-ammonium acetate precipitation protocol described by Lee and Rasheed (1990). For transfection plasmids were prepared as above followed by cesium chloride centrifugation purification. DNA was collected and butanol-extracted to remove ethidium bromide. It was then sterile filtered and quantitated.

Transfection and Subcellular Localization

DAP.3 cells (3×10^5) were cultured on coverslips in a 35-mm well of a six-well culture plate for 20–24 h. PBPHA, PBPHA Δ pre, or

pBKCMV DNA (1 μ g/well) were added to OptiMEM media (GIBCO-BRL) (total 100 μ l/well) and mixed with a LipofectAmine (GIBCO-BRL)-OptiMEM cocktail (9 μ l LipofectAmine plus 91 μ l OptiMEM/well, using 100 μ l cocktail/well) at room temperature for 15–30 min. OptiMEM (0.8 ml/well) was added to DNA/LipofectAmine/OptiMEM solution, mixed, and layered on cells (1 ml/well) already washed three times with DMEM or OptiMEM. The transfection media was left on the cells at 37°C in an atmosphere of 5% CO₂ for 5 h and replaced with 10% CM (2 ml). After ~48 h posttransfection, cells were examined by confocal immunofluorescent microscopy as described (Domanico *et al.*, 1993). Cells were stained with mAb 12CA5 specific for the influenza HA epitope in the presence or absence of HA peptide (0.1 mg/ml) and detected using fluoresceinated F(ab')₂ sheep antibodies (Vector Laboratories, Burlingame, CA).

In Vitro Import into Isolated Yeast Mitochondria

Isolation of mitochondria was as described by Stuart *et al.* (1994). The cDNA encoding PBP74 was subcloned into pGem11z under the control of the SP6 RNA polymerase promoter. The plasmid, termed pG11z-PBP1.8, was generated by subcloning a gel-purified *Xba* I-*Bam*HI fragment from pG7z-PBP1.8 into pGem11z. To ensure efficient translation, the distance between transcription initiation and the initiator ATG was shortened to 41 nucleotides, and the resulting clone was termed pG11z-PBP1.8.1. Radioactively-labeled precursor of PBP74 was synthesized in vitro by transcription/translation in the presence of ³⁵S-Met using the coupled-reticulocyte lysate kit. All other precursors (i.e., *Neurospora crassa* β -subunit of F₁-ATPase and *S. cerevisiae* cytochrome *c* heme lyase) were translated according to the supplier's procedures in reticulocyte lysate (Promega). Unless stated otherwise, import reactions were performed in import buffer (0.6 M sorbitol, 20 mM 3-(*N*-morpholino)propanesulfonic acid pH 7.2, 50 mM KCl, 10 mM MgCl₂, 2.5 mM EDTA, 2 mM KH₂PO₄, and 30 mg/ml fatty acid free bovine serum albumin [BSA]) in the presence of 2 mM ATP and 3 mM NADH (Glick, 1991). Forty-five micrograms mitochondria and 2–5 μ l reticulocyte lysate containing the preproteins were used per sample in a total volume of 100 μ l. Import was allowed for 20 min at 25°C. After chilling on ice, proteinase K was added at 60 μ g/ml, and samples were incubated for 15 min on ice. Proteolysis was halted by adding 2 mM phenylmethylsulfonyl fluoride (PMSF) from a 200 mM stock solution in ethanol. After addition of 1 ml SoH buffer (0.6 M sorbitol and 20 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid [HEPES] pH 7.2), mitochondria were reisolated and washed involving centrifugation for 7 min at 6000 $\times$ g (JA18.1 Beckman rotor, Fullerton, CA), resuspension in 0.1 ml SoH buffer, addition of 1 ml SoH buffer, and another centrifugation for 12 min at 10 000 $\times$ g. The mitochondrial pellet was dissolved in sample buffer, and proteins were separated by SDS-PAGE. Radioactive proteins were visualized by fluorography of the resulting gels. Quantitation was by laser scanning densitometry (Pharmacia Ultrascan GSXL, Piscataway, NJ).

Subcellular Fractionation and Purification of Mitochondria

³⁵S-Met-labeled CH27 cells were homogenized, and the homogenate was centrifuged to remove nuclei and unbroken cells as previously detailed (Qiu *et al.*, 1994). The postnuclear supernatant was then subjected to centrifugation at 10 000 $\times$ g to pellet mitochondria. The pellet contained >85% of the enzyme activity of the mitochondrial marker, cytochrome *c* oxidase (cyt *c* ox), and the remainder of the activity is detected in the supernatant. The supernatant contained the majority of the ER, plasma membrane, Golgi, lysosomes, and early and late endosomes as described elsewhere (Qiu *et al.*, 1994). The mitochondria in the pellet were resuspended in 1 ml homogenization buffer and layered onto a 10-ml Percoll gradient (1.05 gm/ml) and then centrifuged as described previously (Qiu *et al.*, 1994). Gradient fractions were collected and assayed for cyt *c* ox activity and β -hexosaminidase (β -Hexos) activity, a marker for lysosomes and endosomes, as described (Qiu *et al.*, 1994). Fractions were pooled,

and 100- μ l samples were incubated at 4°C for 15 min in homogenization buffer in the presence or absence of proteinase K (0.3 mg/ml) in the presence or absence of 1% NP40. At the end of 15 min, 100 μ l lysis buffer (1% NP-40, 50 mM Tris(hydroxymethyl)aminomethane [Tris]-HCl pH 7.4, 0.15 M NaCl, 0.02% NaN₃) containing 1 mM PMSF was added. The samples were clarified by centrifugation and immunoprecipitated as described (Domanico *et al.*, 1993) using rabbit antisera specific for the C-terminus of PBP74 residues 615–633 or preimmune rabbit serum as a control.

Pulse Chase Experiments and Carbonyl Cyanide *m*-Chlorophenylhydrazone (CCCP) Treatment

CH27 cells (10⁷ cells/ml) were incubated in Met⁻ 5% CM without β -mercaptoethanol for 1 h at 37°C in a 5% CO₂ atmosphere, pulsed with ³⁵S-Met (200 μ Ci/ml) for 3 min, washed and incubated for various times up to 1 h. When indicated, 20 μ M CCCP (Calbiochem, San Diego, CA) was added 20 min before ³⁵S-Met pulsing. Cells were washed and then lysed in RIPA buffer (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 1 mM PMSF), and the lysate was immunoprecipitated with rabbit antisera specific for the N-terminus of PBP74 residues 1–16 or the hsp60-specific mAb II-13 using GammaBind Plus Sepharose (Pharmacia).

RESULTS

Localization of PBP74 by Confocal Immunomicroscopy

Antibodies were raised in rabbits to the predicted C-terminal peptide of PBP74 (residues 615–633) and affinity purified on a PBP74 615–633-Sepharose column. Residues 615–633 are unique to PBP74 and distinguish PBP74 from other hsp70 family members (Domanico *et al.*, 1993). As shown, the antibodies immunoprecipitated a 74-kDa protein from lysates of ³⁵S-labeled L cells that migrated at the molecular weight of peptide-affinity purified PBP74 (Figure 1A). By confocal immunofluorescence microscopy the antibodies showed a clear mitochondrial staining pattern that was indistinguishable from the pattern obtained using a mitochondrial hsp60 specific mAb (Figure 1B). Staining with antibodies directed against the C-terminus of PBP74 was specifically inhibited by the C-terminal peptide used for raising the antiserum but not by the unrelated N-terminal peptide (Figure 1C). Thus, by this criterion PBP74 is present in mitochondria.

Subcellular Localization of a Recombinant Epitope-tagged PBP74 in Transient Transfectants

To rule out possible cross-reactivities of our antibody preparation, an epitope tag encoding a nine-amino acid sequence derived from the influenza HA (Wilson *et al.*, 1984) was inserted into the C-terminal region of PBP74 between the coding sequences for residues 614 and 615 (Figure 2) (Kolodziej and Young, 1991). The site for insertion of the HA tag in the C-terminal region was based on an alignment of PBP74 and a *Drosophila melanogaster* homologue, Drohsc5a (Rubin *et al.*, 1993). Drohsc5a and PBP74 are over 75% identical. However, Drohsc5a

contains a nine-residue segment not present in PBP74 (Figure 2), suggesting that PBP74 would accommodate an insertion of the HA-epitope at this site. The HA epitope-tagged cDNA plasmid was termed pB-PBPHA. The protein products of pB-PBPHA were analyzed *in vitro*. After transcription and translation of pB-PBPHA *in vitro*, the products were subjected to immunoprecipitation with the HA-specific mAb 12CA5 (Figure 3). A protein in the 70-kDa range was specifically immunoprecipitated (Figure 3, lane 5). Immunoprecipitation was inhibited in the presence of excess HA peptide (Figure 3, lane 8). The 70-kDa translation product was also recognized by the antisera specific for the C-terminus of PBP74 (Figure 3, lane 6), whereas immunoprecipitations with normal rabbit serum yielded no products (Figure 3, lane 9). Both the PBP74 C-terminal specific antisera and the HA-specific mAb recognized all major bands from the *in vitro* transcription and translation reaction mixture. This was in contrast to rabbit antisera specific for the N-terminus of PBP74 that immunoprecipitated only the 70-kDa product (Figure 3, lane 4), suggesting that the smaller products result from initiation of translation at internal methionines.

For expression in L cells, the coding region of the epitope-tagged PBP74 cDNA was cloned into the eukaryotic expression vector pBKCMV yielding the vector PBPHA. In addition, to allow an analysis of the contribution of the PBP74 presequence to localization, the region encoding the presequence was removed leaving only the coding sequence for the mature PBP74 protein. This vector was termed PBPHA Δ pre. After transfection into L cells, PBPHA and PBPHA Δ pre were transiently expressed, and the subcellular location of the encoded proteins was investigated by confocal immunofluorescence microscopy using the HA-specific mAb. L cells expressing PBPHA show a clear mitochondrial staining pattern (Figure 4A). The pattern is reminiscent of that obtained using the PBP74 C-terminal-specific antibodies (Figure 1B). In contrast, the staining pattern obtained for cells expressing PBPHA Δ pre was not mitochondrial and rather appeared to be cytoplasmic (Figure 4B). For both PBPHA and PBPHA Δ pre the staining of the transfectants was specific in that no staining was detected using an isotype control mAb, and staining was not detected in the presence of excess HA peptide. Likewise, cells transfected with the expression vector alone showed no specific staining. Taken together, these results support the finding from above of a mitochondrial localization of PBP74. Furthermore, the presequence of PBP74 is necessary for correct mitochondrial localization.

Localization of PBP74 by Subcellular Fractionation

As a biochemical approach for the localization of PBP74, mitochondria were purified from cells by Percoll density gradient centrifugation (Figure 5A). Mitochondria sed-

Figure 1. Antibodies specific for the C-terminus of PBP74 yield a mitochondrial staining pattern that overlaps with that of a mAb specific for mt hsp60. (A) Lysates prepared from ^{35}S -Met-labeled L cells were immunoprecipitated as described (Domanico *et al.*, 1993) using peptide-affinity purified antibodies specific for PBP74 residues 615–633 (lane 1) or normal rabbit IgG (lane 3). Lane 2 contains peptide-binding proteins affinity purified from lysates prepared from ^{35}S -labeled CH27 cells as previously described (Domanico *et al.*, 1993). Samples were subjected to 10% SDS-PAGE and fluorography. (B) Immunofluorescence confocal micrograph of L cells stained using peptide-affinity purified rabbit antibodies specific for PBP74 residues 615–633 detected using biotinylated goat antibodies specific for rabbit Ig and Texas red streptavidin (left) or stained using the mt hsp60 specific mAb II-13 detected with FITC-goat F(ab')₂ specific for mouse antibodies (right). Staining and immunofluorescence confocal microscopy was as previously described (Domanico *et al.*, 1993). Bar, 20 μm . (C) Immunofluorescent micrographs of L cells stained with peptide-affinity purified antibodies specific for PBP74 residues 615–633 alone (left), in the presence of 100 molar excess of a peptide representing the C-terminus of PBP74 residues 615–633 (middle), or of an unrelated peptide representing the N-terminus of PBP74 residues 1–16 (right). Staining and immunofluorescence microscopy was as previously described (Domanico *et al.*, 1993). Bar, 3 μm .

imented in two regions of the gradient. Fractions 7–9 contained cytochrome *c* oxidase activity and negligible activity for β -hexosaminidase, an enzyme marker for endosomes and lysosomes, and thus were used for this analysis. PBP74 was immunoprecipitated from purified mitochondria using rabbit antisera specific for the C-terminus of PBP74 615–633 (Figure 5B). Significantly, the PBP74 protein was protected from digestion by externally added protease in the absence of detergent indicating that the protein resides within the mitochondria. Thus, according to two independent techniques, namely confocal immunofluorescence microscopy and cell fractionation, PBP74 is a resident of mitochondria. To study the requirements for the localization of PBP74 to mitochondria, its import into the organelle *in vivo* and *in vitro* was investigated in some detail.

Import of PBP74 into Purified Mitochondria *In Vitro*

To characterize the import of PBP74 *in vitro*, we took advantage of the well-characterized protein import system developed for yeast mitochondria (Glick, 1991). The precursor of PBP74 was synthesized *in vitro* in a coupled transcription/translation system in the presence of ^{35}S -Met. An import reaction was performed by incubating radioactively-labeled precursor with isolated mitochondria for various times at 0 or 25°C (Figure 6A). A substantial fraction of the precursor of PBP74 (lane 8) was processed to a smaller protein, the mature form of PBP74 (lane 7). The majority of the mature PBP74 product, but not of the precursor, was protected from proteolysis by added proteinase K (lane 5) and was only degraded after detergent lysis of the mitochondria

Figure 2. Influenza HA-epitope tagging of PBP74. An alignment of the C-terminal protein sequence of PBP74 with a *D. melanogaster* homologue, Drohac5a (Rubin *et al.*, 1993) shows a nine-amino acid segment not present in PBP74. A nine-amino acid sequence derived from influenza HA was inserted at this site.

(lane 6). The import of PBP74 into mitochondria was time- and temperature-dependent (Figure 6, lanes 1–5). Both the efficiency and the rate of import of PBP74 were comparable to that of a control preprotein, namely the β -subunit of F_1 -ATPase ($F_1\beta$) precursor protein. These data suggest that the precursor of PBP74 is imported into mitochondria and processed to its mature form by the matrix-processing peptidase.

We next addressed whether protease-sensitive surface receptors of the mitochondrial outer membrane were required for import of PBP74. Mitochondria were pretreated with increasing amounts of trypsin. After inactivation of the protease by soybean trypsin inhibitor, an import reaction was carried out using the radioactively-labeled precursors of PBP74 and $F_1\beta$. For both proteins import and processing to the mature forms was reduced significantly after trypsin pretreatment of mitochondria (Figure 6B). The import of the precursor of $F_1\beta$ into trypsin-treated mitochondria was almost completely abolished. In contrast, the import of the precursor of PBP74 into trypsin-treated mitochondria was reduced by only 50%. A similar receptor-independent translocation occurring at reduced efficiencies, so-called “bypass” import, has been observed for a number of preproteins (Pfaffler *et al.*, 1989). It was suggested that preproteins circumvent interaction with the mitochondrial surface receptors by entering the translocation machinery at a later step (Pfaffler *et al.*, 1988).

Import of most mitochondrial preproteins is dependent on the presence of a membrane potential across the inner membrane. The membrane potential of mitochondria was depleted by the addition of an ionophore, valinomycin, and specific inhibitors of the electron transport chain, antimycin A, and of F_1 -ATPase, oligomycin. No significant import of the precursors of PBP74 and of $F_1\beta$ was detected (Figure 7). A similar observation was made when CCCP, a protonophore that abolishes the proton gradient across the inner membrane, was present during the import reaction. In contrast, cytochrome *c* heme lyase, which is imported into the intermembrane space of mitochondria independently of a membrane potential (Lill *et al.*, 1992; Steiner and Lill unpublished results), was virtually unaffected in its import efficiency. Thus, the precursor of PBP74 requires the inner membrane for efficient translocation into mitochondria.

Import into the mitochondrial matrix usually requires matrix ATP, which likely is required for the function of

the matrix mitochondrial hsp70 protein in yeast (Wachter *et al.*, 1992). Substantial depletion of the ATP levels in the matrix space can be achieved by simultaneously inhibiting the F_1F_0 -ATPase by oligomycin and the ADP/ATP translocator by carboxyatractylosid (CAT), whereas intermediate levels of matrix ATP may be obtained by adding oligomycin alone (Stuart *et al.*, 1994). The precursor of PBP74 required ATP for its import into mitochondria because import was not achieved in the presence of oligomycin and CAT (Figure 8). This finding suggests that Ssc1p, the yeast mitochondrial hsp70, is required for the import of the mammalian hsp70. Consistent with this observation, Ssc1p has been reported to be required for its own import (Kang *et al.*, 1990).

To determine the submitochondrial location of PBP74 within the mitochondria, mitochondria were subjected to a swelling procedure that selectively opens the outer membrane, exposing proteins of the intermembrane space to added proteases. The matrix proteins in swollen mitochondria remain protected from proteolytic attack. After swelling of the mitochondria, imported PBP74 was protected from proteolysis (Figure 9). A similar result was obtained for imported $F_1\beta$ as well as for endogenous, matrix-localized Mpi1p (Figure 9). In contrast, the levels of endogenous components of the intermembrane space, cytochrome c_1 heme lyase or cytochrome b_2 , were substantially reduced after swelling and addition of proteinase K, indicating the selective opening of the outer membrane. Taken together, the results of the *in vitro* import studies show that the precursor of PBP74 can efficiently become translocated into the matrix space, where its signal sequence is cleaved. The requirements for import are similar to those estab-

Figure 3. SDS-PAGE analysis of the transcription and translation of influenza HA-epitope-tagged PBP74 *in vitro*. The *in vitro* transcription-translation products of pB-PBPHA (lane 2) were immunoprecipitated using the following: the HA-specific mAb 12CA5 alone (lane 3), mAb 12CA5 in the presence of an excess of the HA peptide (lane 4), rabbit antisera specific for the N-terminus of PBP74 residues 1–16 (lane 5), rabbit antisera specific for the C-terminus of PBP74 residues 615–633 (lane 6), and normal rabbit serum (lane 7). The *in vitro* transcription-translation products of the untagged plasmid, pG7z-PBP1.8 were immunoprecipitated using rabbit antisera specific for the C-terminus of PBP74 (lane 9) or normal rabbit serum (lane 10). A sample of an *in vitro* transcription-translation reaction that contained no DNA is shown (lane 1). MW markers are shown (lane 3). All samples were subjected to SDS-PAGE and fluorography.

Figure 4. Subcellular localization of HA-epitope-tagged PBP74 in transient transfections. L cells were transfected with PBP74 (A) or PBP74 Δ pre (B) as described in MATERIALS AND METHODS and processed for immunofluorescent microscopy as previously described (Domanico *et al.*, 1993). Cells were stained with mAb 12CA5, specific for the influenza HA-epitope and detected using fluoresceinated F(ab')₂ sheep antibodies specific for rabbit Ig. Shown are representative cells from ~100 transfectants viewed. Bars, 15 μ m (A) and 10 μ m (B).

lished for other matrix-localized proteins (Kiebler *et al.*, 1993; Stuart *et al.*, 1994; Wachter *et al.*, 1992).

PBP74 is Synthesized as a Preprotein in Mammalian Cells and Requires Import into Mitochondria for Its Maturation

To investigate the biogenesis of the PBP74 in mammalian cells, pulse chase analyses were carried out in the presence and absence of the protonophore CCCP. CH27 were pulsed with ³⁵S-Met and chased for various times after which PBP74 was immunoprecipitated. At early time points both the precursor of PBP74 and the mature protein were detected (Figure 10A). Within 5–20 min the precursor was largely converted into the mature form indicating import into mitochondria. To determine if the conversion of the preprotein to the mature protein is dependent on a membrane potential, CH27 cells were preincubated before the pulse labeling in the presence of CCCP. Only the preprotein was immunoprecipitated even after a 60-min chase period (Figure 10A). Similarly, only the precursor form of the mitochondrial hsp60, as a control, was immunoprecipitated in the presence of CCCP (Figure 10B). Thus, in agreement with the findings from *in vitro* import studies above, the processing of the PBP74 preprotein to its mature form is dependent on a membrane potential in CH27 cells *in vivo*, indicating that import into mitochondria is required for maturation of PBP74.

DISCUSSION

The results of studies presented here demonstrate that the product of a recently cloned cDNA, PBP74, encoding a new member of the mammalian hsp70 family is a mitochondrial matrix protein. The majority of mitochondrial proteins are encoded by nuclear genes and must be transported into the mitochondria after translation in the cytosol. Progress over the last several years

has provided considerable molecular details of mechanisms by which proteins destined for the mitochondria are imported and then sorted to the appropriate sub-compartments within the mitochondria, namely the

Figure 5. PBP74 is enriched in purified mitochondria and resides in a protease protected location. (A) Percoll gradient fractionation of ³⁵S-Met-labeled CH27 cell homogenate. Gradient fractions were collected and assayed for cyt c ox activity and β -Hexos activity. The insert shows the gradient density measured by density marker beads. (B) Pool gradient fractions 7–9 were incubated at 4°C for 15 min in the presence or absence of proteinase K (Prot K) in the presence or absence of NP40. Samples were subsequently immunoprecipitated with rabbit antisera specific for the C-terminus of PBP74 residues 615–633 (Anti-C) or with preimmune sera (Pre). All samples were subjected to SDS-PAGE and fluorography.

Figure 6. The precursor of PBP74 is imported into isolated mitochondria and becomes processed to its mature form. (A) Mitochondria and radioactively-labelled precursors of PBP74 (PBP74) and the β -subunit of F_1 -ATPase ($F_1\beta$) were mixed in import buffer supplemented with 2 mM ATP and 3 mM NADH and incubated for the indicated times at 0 or 25°C. After chilling on ice, samples were treated with proteinase K where indicated. The sample in lane 6 contained 0.2% Triton X-100 to lyse the mitochondria. After 15 min on ice, 2 mM PMSF was added. Proteins were precipitated with trichloroacetic acid (TCA) and analyzed by SDS-PAGE and fluorography. Lane 8 contains 10% of the input precursor proteins as a standard (std.). (B) Mitochondria were treated with trypsin for 15 min on ice in import buffer containing 1 mg/ml BSA. Proteolysis was halted by the addition of 1 mg/ml soybean trypsin inhibitor. After 5 min on ice, samples were supplemented with 2 mM ATP and 3 mM NADH, and import reactions were carried out for 20 min at 25°C as described in A. Samples were treated with proteinase K, where indicated mitochondria were reisolated by centrifugation and imported proteins analyzed by SDS-PAGE and fluorography. Precursor form (p) and mature form (m) are indicated.

outer and inner membranes, the intermembrane space, and the matrix (reviewed in Kiebler *et al.*, 1993). The precursor forms of nuclear-encoded mitochondrial proteins contain targeting and sorting signals that for proteins destined for the latter three compartments usually are encoded as cleavable amino-terminal signal sequences. The isolation of the PBP74 cDNA indicated that it encodes a 46-residue presequence not present in the mature protein (Domanico *et al.*, 1993). The presequence was similar to mitochondrial-targeting sequences that may form amphipathic α helices but did not conform to this motif completely (von Heijne, 1986). Here we show that the presequence is required for mitochondrial localization, and in its absence PBP74 remains in the cytosol. The presequence is removed upon import into isolated yeast mitochondria *in vitro*. In mammalian cells removal of the presequence also

required import into mitochondria as shown by the accumulation of the preprotein in cells treated with the protonophore CCCP, which destroys the proton gradient across mitochondrial inner membranes. In general, the signal sequences of the precursor proteins interact with proteins on the surface of the mitochondria that initiate their transport across the mitochondrial outer membrane. Here we show that the transport of PBP74 into isolated yeast mitochondria is not absolutely dependent on protease-sensitive mitochondrial surface proteins but that such proteins increase the efficiency of import. Such receptor-independent translocation into the mitochondria, referred to as bypass import, has been described for a number of mitochondrial proteins (Pfaffler *et al.*, 1989). Transport across the inner membrane requires a membrane potential, $\Delta\Psi$, and is usually coupled to completion of translocation across the outer membrane. For import into the matrix space, ATP hydrolysis catalyzed by mitochondrial hsp70 is required (Kang *et al.*, 1990; Wachter *et al.*, 1992). Here we show that the translocation of PBP74 into the matrix of isolated yeast mitochondria requires a membrane potential and matrix ATP. In mammalian cells maturation of the preprotein is blocked by depolarization of the membrane potential of the mitochondrial inner membrane by the protonophore CCCP. The imported precursor proteins associate with additional matrix proteins that facilitate the completion of transport of the entire peptide chain across the membrane, remove the signal sequence, and promote the appropriate folding of the protein into its final active form or the relocation across the inner membrane. In yeast, the mt hsp70 both facilitates the movement of the precursor protein into the matrix and

Figure 7. Import of PBP74 into mitochondria requires a membrane potential across the inner membrane. Isolated mitochondria in import buffer were treated for 1 min on ice with AOV, a mixture of antimycin A (4 μM), oligomycin (10 μM), and valinomycin (0.25 μM), or with increasing amounts of CCCP. Import reactions were carried out using a mixture of the precursors of cytochrome *c* heme lyase (CCHL), $F_1\beta$, and PBP74 described in Figure 6B. The resulting fluorographs were quantitated by laser scanning densitometry. Results are given relative to the import in the absence of inhibitors.

Figure 8. Import of PBP74 into mitochondria requires matrix ATP. Isolated mitochondria in import buffer were treated with 20 μ M oligomycin (Oli) and 20 μ M carboxyatractylid (CAT) for 3 min at 25°C (Stuart *et al.*, 1994). After supplementing the samples with 2 mM ATP and 3 mM NADH, import reactions were performed, analyzed, and quantitated as described in Figure 7. Results are given relative to the import in the absence of inhibitors.

prevents inappropriate aggregation of the imported polypeptide chain for either folding or for relocation (Kang *et al.*, 1990; Ostermann *et al.*, 1990; Scherer *et al.*, 1990; Manning-Krieg *et al.*, 1991; Gambill *et al.*, 1993; Voos *et al.*, 1993). Thus, by analogy, PBP74 presumably interacts with PBP74 resident in the matrix that facilitates its own import.

A mammalian mt hsp70 protein has been described (Leustek *et al.*, 1989; Mizzen *et al.*, 1989). Mizzen *et al.* (1989) showed by a variety of biochemical and immunological criteria that a glucose-regulated protein of 75 kDa, which they termed grp75, is a mammalian mt hsp70. Grp75 was shown to reside in the mitochondria by immunofluorescence staining using polyclonal rabbit antisera raised to grp75. By Western blotting, grp75 was found in highly purified preparations of mitochondria in a protease-protected location. Moreover, grp75 was shown to accumulate as a larger precursor in the presence of nonactin, a potassium ionophore that inhibits the processing of cytoplasmic precursor proteins destined for the mitochondria. Leustek *et al.* (1989) similarly described a mammalian mt hsp70 using subcellular fractionation. Here we provide evidence that PBP74 cDNA encodes a mammalian matrix mt hsp70 that is likely grp75.

We previously showed that peptide-affinity purified antibodies to a peptide representing the N-terminus of PBP74 showed a predominant vesicular and a weak mitochondrial-staining pattern. Here we show that peptide-affinity purified antibodies raised to a peptide representing the C-terminus of PBP74 shows a strong mitochondrial-staining pattern. We have recently generated and characterized several mAbs derived from spleens of mice immunized with the same peptide representing the N-terminus of PBP74 (Green, Gu, Ifkovits, Kaumaya, and Pierce, unpublished results) that may explain these differing results. Among these mAbs we

identified one that cross-reacts with PBP74, BiP/grp78, and hsc 70 in Western blot. However, by confocal immunofluorescence microscopy the mAb yields a BiP/grp78-specific staining, similar to the vesicular staining obtained using the N-terminal-specific rabbit antibodies. mAbs were also generated from spleens of mice immunized with the N-terminal peptide that were highly specific for PBP74 and resulted in a mitochondrial staining pattern. We conclude that the previously published vesicular/mitochondrial pattern represents the composite of possible reactivities obtained after immunization with the N-terminal peptide of PBP74. The exclusive mitochondrial staining pattern shown here, obtained using rabbit antibodies specific for the C-terminus of PBP74, is consistent with the mitochondrial-staining pattern obtained by Michikawa *et al.* (1993b) using a mAb specific for an epitope present in the C-terminus of PBP74. However, these results do not resolve the contradictory findings of Wadhwa and colleagues (Wadhwa *et al.*, 1993a,b; Kaul *et al.*, 1993) who showed mortalin to be present in the cytosol and in a perinuclear location.

Figure 9. PBP74 is imported into the mitochondrial matrix. Import reactions for the precursors of PBP74 and F₁ β were performed as described in Figure 6B and terminated by the addition of trypsin (50 μ g/ml). After 15 min on ice and addition of soybean trypsin inhibitor (1 mg/ml), mitochondria were pelleted and resuspended in 50 μ l SoH buffer containing 1 mg/ml soybean trypsin inhibitor. To induce swelling of the mitochondria, samples were diluted with 450 μ l HP buffer (20 mM HEPES pH 7.2 and 60 μ g/ml proteinase K) containing 0.6 M sorbitol (-), 0.06 M sorbitol (+), and 0 M sorbitol (++) followed by an incubation on ice for 15 min. Proteolysis was stopped by the addition of 2 mM PMSF, and mitochondria were resuspended. After resuspending pellet in 20 mM HEPES pH 7.2, proteins were precipitated by TCA, resolved by SDS-PAGE, and blotted onto nitrocellulose. Import of radiolabeled PBP74 and F₁ β was quantified by autoradiography of the blot, and endogenous unlabeled proteins were analyzed by immunostaining (enhanced chemiluminescence detection system) using antibodies directed against Mpi1p, cytochrome c₁ heme lyase (CC1HL), or cytochrome b₂ (Cyt b₂).

Figure 10. CCCP blocks the maturation of PBP74 in CH27 cells. Pulse chase analyses were carried out in the presence or absence of CCCP. Samples were immunoprecipitated using PBP74 specific antisera (A) or the hsp60-specific mAb, II-13 (B). Immunoprecipitates were subjected to 10% SDS-PAGE and fluorography. Indicated are the precursor proteins (P) and the mature proteins (M).

Our interest in members of the hsp70 family that ultimately lead to the cloning of the cDNA encoding PBP74 stemmed from studies of the potential role of molecular chaperones in the immunological function termed antigen processing (reviewed in DeNagel and Pierce, 1992, 1993). Antigen processing involves the internalization of antigen into cells that express the major histocompatibility complex (MHC) class II molecules, proteolysis of the antigen, and binding of the resulting peptides to the MHC molecules. We hypothesized that molecular chaperones may be involved in the scavenging of peptides for subsequent binding to MHC class II molecules or in the assembly process by which peptides bind to MHC class II molecules (DeNagel and Pierce, 1992). PBP74 was originally isolated and characterized in this laboratory as one of at least three peptide-binding proteins obtained from immune cells by virtue of their ability to bind to a peptide column but not to a column prepared from the corresponding native protein (Lakey *et al.*, 1987). This mixture of proteins was collectively referred to as PBP72/74. Subsequent serological and protein sequence analyses showed the individual members of PBP72/74 are related to the hsp70 family (VanBuskirk *et al.*, 1989). PBP72/74 minimally contained BiP/grp78, the ER resident protein, and PBP74, a new member of the mammalian family, and possibly hsc70 (Domanico *et al.*, 1993). The link between these hsps and antigen processing was the observation that antibodies raised in rabbits to PBP72/74 blocked antigen processing (Lakey *et al.*, 1987; VanBuskirk *et al.*,

1991). The antisera was shown by flow cytometry and by immunoelectron microscopy to bind to target proteins on the plasma membrane, in early endocytic vesicles containing MHC class II molecules, to Golgi, and to small cytoplasmic vesicles (VanBuskirk *et al.*, 1991). Thus, the original antisera raised to the mixture of peptide-binding proteins did not show reactivity to mitochondrial PBP74 or to the ER, BiP/grp78. The antibodies presumably blocked processing by interfering with the peptide-MHC class II molecule assembly process either at the plasma membrane or in early endosomes. Insights as to the identity of the hsp70 that may play a role in antigen processing have come from our recent success in isolating the subcellular vesicles in which peptides first bind to MHC class II molecules (Qiu *et al.*, 1994). Our preliminary results indicate that a 74-kDa protein, distinct from PBP74, is immunoprecipitated from these vesicles using the antisera raised to PBP74. Experiments are in progress to further characterize and identify this hsp70 family member and its potential role in antigen processing.

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