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PURIFICATION OF 52 kDa PROTEIN: A PUTATIVE COMPONENT OF THE IMPORT MACHINERY FOR THE MITOCHONDRIAL PROTEIN-PRECURSOR IN RAT LIVER

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Summary: A protein having a molecular mass of 52 kDa was purified to homogeneity from solubilized mitochondrial membrane proteins by affinity column chromatography using the synthetic presequence of ornithine aminotransferase (OAT) as the ligand. This 52 kDa protein was specifically bound to the affinity column and eluted with 1 mM OAT-presequence, indicating that it recognized the presequence and bound to it specifically. Anti-52 kDa protein Fab fragments specifically inhibited the import of OAT-precursor into mitochondria, showing that the 52 kDa protein plays an essential role in this process. These results suggest that 52 kDa protein is a component of the import machinery of the mitochondrial protein-precursor in the mitochondrial membrane.

The mitochondrial protein-precursors synthesized on cytoplasmic polysomes are transported into mitochondria post-translationally (1, 2). It has been shown that ATP and an inner membrane potential ($\Delta\Psi$) are required as energy for this process (2). Furthermore, there are also several reports on factors that are essential in the course of translocation of the mitochondrial proteins, and some of these factors have been purified from the cytosol and the mitochondrial membrane (3-13).

In the previous papers, we reported the purifications of a 28 kDa protein from rabbit reticulocyte lysate (5) and a 29 kDa protein from rat liver mitochondrial membranes (13) and showed that these two proteins play essential roles in the transport of mitochondrial proteins. We purified these two proteins on an affinity column prepared with a chemically synthesized peptide composed of 54 amino acids containing the presequence of ornithine aminotransferase (OAT) at its NH_2-terminal as a ligand (5, 13). Furthermore, we reported that a 52 kDa protein was co-purified with the 29 kDa protein from crude solubilized mitochondrial membrane proteins, although we did not determine whether this 52 kDa protein has any role in the import of mitochondrial proteins (13).

In the present paper, we report that the 52 kDa protein was specifically eluted with the synthetic OAT-presequence (composed of 34 amino acids) from an affinity column and that anti-52 kDa protein antibody specifically inhibited the

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import of the OAT-precursor into mitochondria. These findings indicate that the 52 kDa protein is also an essential component of the import machinery of mitochondrial protein in the membrane.

MATERIALS AND METHODS

SYNTHESIS OF PEPTIDES AND PREPARATION OF PEPTIDE-CONJUGATED AGAROSE: The two peptides used in this study, a 54 amino acids peptide (54-AA peptide) and the OAT-presequence, were chemically synthesized by Applied Biosystems Japan Co.(Tokyo, Japan). The amino acid sequence of the 54-AA peptide is the same as that from Met 1 to His 53 of the OAT-precursor reported by Mueckler and Pitot (14), with an additional C-terminal Cys 54 to facilitate chemical crosslinking, as described in our previous paper (13). The 52 kDa protein was purified from solubilized mitochondrial membrane proteins with an affinity column of ω-aminobutyl-agarose conjugated with 54-AA peptide by using m-maleimidobenzoyl-N-hydroxysuccinimide ester (13).
OTHER METHODS: Precursors of mitochondrial proteins were synthesized in a cell-free translation system using total rat liver RNA and a nuclease-treated reticulocyte lysate as described previously (15). After the translation reaction, the reaction mixture was centrifuged for 30 min at 320,000 xg to remove ribosomes and the supernatant was used as a source of mitochondrial protein-precursors for import into mitochondria (15). SDS-polyacrylamide slab gel electrophoresis was performed by the method of Laemmli (16). Fab fragments were prepared from IgG by the method of Mage (17). Ornithine aminotransferase and porin were purified as described previously (18, 19) and antibodies against these enzymes and the 52 kDa protein were prepared by immunizing rabbits with these purified proteins as described previously (20).
MATERIALS: [^{35}S] Methionine (>800 Ci/mmol) was obtained from American Radiolabeled Chemicals, Inc., USA. ATP-agarose (A 9264) and ω-aminobutyl-agarose (A 6142) were purchased from Sigma Chemical Co.. Nikkol (octaethyleneglycol mono-n-dodecyl ether) was from Nikko Chemicals (Tokyo, Japan), and the silver staining kit was from Kanto Chemical Co. (Tokyo, Japan).

RESULTS AND DISCUSSION

PURIFICATION OF 52 kDa PROTEIN: Crude outer mitochondrial membranes and a solubilized protein fraction of mitochondrial membranes were prepared as described previously (13). The solubilized proteins (2,210 mg) were applied to an ATP-agarose column and the unadsorbed fraction was applied to a 54-AA peptide conjugated agarose column as described previously (13). The column was washed with buffer A (10 mM Hepes-KOH, 2 mM magnesium acetate, 2 mM GSH, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 10% glycerol) (pH 7.6) containing 800 mM potassium acetate and 1% Triton X-100, and then with buffer A containing 200 mM potassium acetate and 1% Nikkol. The proteins bound to the column were eluted with buffer A containing 200 mM potassium acetate, 1% Nikkol, and 1 mM OAT-presequence. Elution of the affinity column with OAT-presequence yielded only the 52 kDa protein (about 0.8 mg), indicating that this protein recognized and specifically bound to the OAT-presequence (Fig. 1, lane 2). The 29 kDa protein was eluted together with 52 kDa protein with chaotropic ion (Fig. 1, lane 1) (13), but it was not eluted with the synthetic OAT-presequence, suggesting that

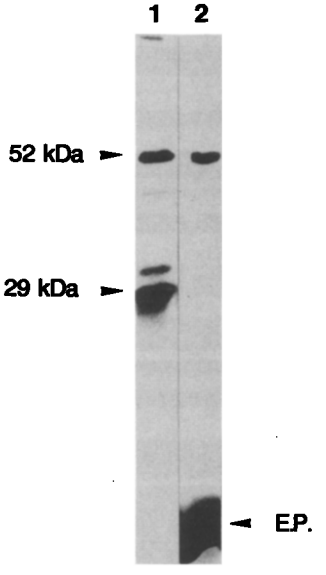

FIG. 1. Electrophoretogram of the purified 52 kDa protein. A solubilized protein fraction prepared from mitochondrial outer membranes was partially purified on ATP-agarose. The unadsorbed fraction (2,000 mg) was applied to a 54-AA peptide conjugated agarose column (bed volume 3.3 ml), and adsorbed proteins were eluted with chaotropic ion (3 M $MgCl_2$, lane 1) or 1 mM OAT-presequence (lane 2) as described in the text. Electrophoresis was performed on a 11% polyacrylamide slab gel in the presence of 0.2% SDS. Proteins were detected by silver staining. E.P., OAT presequence.

this 29 kDa protein recognizes both the OAT-presequence and a part of the NH_2-terminal amino acid sequence of the mature OAT contained in the ligand or that it has a greater affinity to the presequence.

These results strongly suggest that the 52 kDa protein is part of the import machinery in the mitochondrial membrane together with the 29 kDa protein and other proteins that have not yet been identified. To confirm this possibility, we prepared anti-52 kDa protein antibody by immunizing a rabbit with the purified 52 kDa protein, as described previously (19).

EFFECT OF ANTI-52 kDa PROTEIN ANTIBODY ON THE IMPORT OF OAT-PRECURSOR INTO MITOCHONDRIA: Treatment of mitochondria with anti-52 kDa protein Fab fragments strongly inhibited the import of the OAT-precursor (Fig. 2A). This inhibition seemed to be caused by specific interaction of the antigen with its antibody, because control Fab fragments prepared from non-immune rabbit and anti-porin Fab fragments did not inhibit this import-reaction (Fig. 2A and B). The present results suggest that the 52 kDa protein is an essential component of the import machinery for the mitochondrial protein-precursor in the mitochondrial outer membrane.

We have reported (5) that a 28 kDa protein, a component of the targeting factor required for the transport of mitochondrial protein-precursors to the mitochondrial surface, could be purified to homogeniety using the same affinity

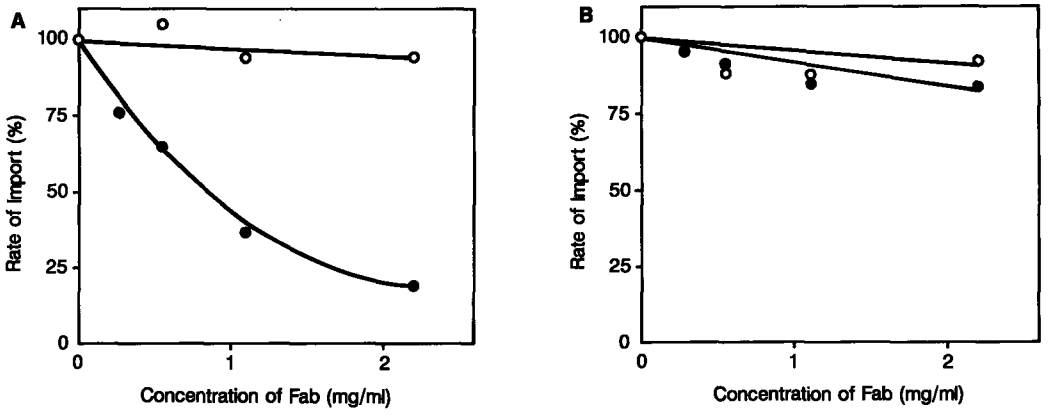

FIG. 2. Effect of anti-52 kDa protein Fab fragments and anti-porin Fab fragments on the import of OAT-precursor. A) Effect of anti-52 kDa protein Fab fragments. Mitochondria (final concentration, 0.25 mg/ml) were incubated with anti-52 kDa protein Fab fragments (•) or control Fab fragments prepared from a non-immune rabbit (o) at the concentrations indicated in the Figure for 15 min at 25°C and then for 90 min at 0°C. Then, each mitochondrial fraction(100 µl, 25 µg) was incubated with 100 µl of cell-free translation products for 15 min at 30°C. The mitochondria were then reisolated and their imported OAT was analyzed (15). Bands of OAT on the fluorogram were quantified by densitometry. B) Effect of anti-porin Fab fragments. Experimental and analytical conditions were as described above. •, treated with anti-porin Fab fragments; o, treated with control Fab fragments.

column as that used for purification of the 52 kDa protein, and that anti-28 kDa protein antibody inhibited the binding of the precursor to the mitochondria at 0°C as well as its import. On the contrary, we found that anti-52 kDa protein antibody had no effect on the binding of the OAT-precursor to the mitochondria at 0°C : on incubation with mitochondria at 0°C for 20 min, the precursor bound to the mitochondria, as shown in Fig. 3, lane 1, and this binding was not effected by pre-treatment of the mitochondria with anti-52 kDa protein antibody (lanes 8

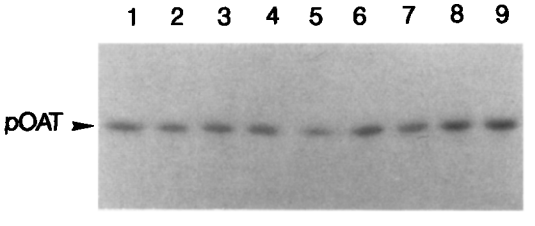

FIG. 3. Effects of anti-52 kDa protein Fab fragments and anti-porin Fab fragments on the binding of pOAT to mitochondria. Mitochondria (final concentration, 0.25 mg/ml) were treated with each antibody as described in the legend for Fig. 2. Then, the samples were incubated with 100 µl of cell-free translation products for 20 min at 0°C. The mitochondria were then reisolated and OAT-precursor recovered from them was assayed by fluorography. Lane 1, untreated; lanes 2-4, treated with control Fab fragments at concentrations of 0.41 mg/ml, 0.83 mg/ml, and 1.7 mg/ml, respectively; lanes 5-7, treated with anti-porin Fab fragments at concentrations of 0.41 mg/ml, 0.83 mg/ml, and 1.7 mg/ml, respectively; lanes 8 and 9, treated with anti-52 kDa protein Fab fragments at concentrations of 0.83 mg/ml and 1.7 mg/ml, respectively.

and 9), control IgG (lanes 2-4) or anti-porin antibody (lanes 5-7). We are now carrying out immunochemical studies on the molecular events occurring during the binding of the precursor-protein to the mitochondria.

As we reported (21) that the OAT-precursor specifically bound to the mitochondrial surface at 0°C and the OAT-precursor bound to the surface was efficiently imported into mitochondria, these results indicate that the 52 kDa protein may not have an essential role in the early binding step of the mitochondrial protein-precursors.

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