Signal sequence region of mitochondrial precursor proteins binds to mitochondrial import receptor

(expression in Escherichia coli / cell-free translation/preproteins/in vitro binding/phosphate translocator)

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ABSTRACT An integral mitochondrial membrane protein (p32) of yeast has previously been molecularly cloned and sequenced and suggested to function as a mitochondrial import receptor. However, this protein has also been proposed to function as phosphate translocator (Guérin, B., Bukusoglu, C., Rakotomana, F. & Wohlrab, H. (1990) J. Biol. Chem. 265, 19736-19741; Phelps, A., Schobert, C. T. & Wohlrab, H. (1991) Biochemistry 30, 248-252). Here we have purified p32 after expression of its gene in Escherichia coli and assayed its ability to bind to various preproteins containing signal sequences for protein translocation into mitochondria, chloroplasts, or the endoplasmic reticulum. Our data suggest that p32 contains a binding site specific for the signal sequence region of mitochondrial preproteins. These data are consistent with the previous assignment of p32 as an import receptor and are discussed with regard to the apparently conflicting assignment of this protein as phosphate translocator.

We have identified (1) a candidate protein for a yeast mitochondrial "import receptor." We have proposed (1) that this import receptor functions as a signal sequence binding unit of a signal-sequence-gated protein-conducting channel in the outer mitochondrial membrane and that this channel, with a signal-sequence-gated channel in the inner mitochondrial membrane, serves as the conduit for protein import into mitochondria. The import receptor was identified by an anti-idiotypic antibody approach. A chemically synthesized signal peptide specifying protein import into mitochondria was used to generate monospecific antibodies. These antibodies were then used as immunogens for a second round of antibody production; the latter antibodies can be expected to be anti-idiotypic (2) and, therefore, to mimic the signal peptide. Indeed, Fab fragments of these antibodies inhibited signal-sequence-mediated protein import into mitochondria, presumably by binding to the signal sequence binding site of the import receptor and by competing with the signal sequence of the protein to be imported. Immunoblots identified an integral membrane protein with an apparent molecular mass of 32 kDa. The anti-idiotypic-antibody-reactive p32 was purified to homogeneity (1). Fab fragments of monospecific antibodies raised against purified p32 also inhibited protein import into mitochondria (1). These data provided anti-idiotypic-antibody-independent evidence that was consistent with the anti-idiotypic-antibody-dependent identification of p32. Immunogold decoration of mitochondria with both anti-idiotypic and anti-p32 antibodies showed that p32 was localized in the outer mitochondrial membrane at sites where the outer and inner mitochondrial membranes are closely apposed (contact sites) (1) and where protein import has been suggested to take place (3, 4).

However, localization of p32 to the inner mitochondrial membrane has not yet been ruled out. Moreover, because of immunodecoration of the outer mitochondrial membrane at contact sites, one cannot be certain whether the observed inhibition of protein import by Fab fragments is specific. For example, if p32 were not directly involved in protein import but nevertheless was localized in the outer membrane at contact sites, binding of Fab fragments might inhibit protein import by nonspecific steric interference. Examples for such nonspecific interference by monospecific Fab fragments have been identified for proteins that had been thought to function in protein translocation across the rough endoplasmic reticulum (RER) (5, 6) and the Escherichia coli plasma membrane (7, 8).

Surprisingly, when p32 was molecularly cloned and sequenced (9), its primary structure was found to be 40% identical to mitochondrial membrane proteins of rat liver (10) and bovine heart (11) that had been proposed to function as phosphate translocators. More recently, a yeast mitochondrial membrane protein that is 100% identical to p32 has been molecularly cloned and sequenced and has also been suggested to function as a phosphate translocator (12, 13). Haploid cells carrying a disrupted gene were unable to grow in nonfermentable carbon sources, indicating that p32 is important for mitochondrial function (9). Based on the available data, the questions arising, therefore, are as follows: Could p32 be a bifunctional protein and serve as both mitochondrial import receptor and phosphate translocator or was either one (or both) function(s) of p32 misidentified? Although out in vitro precursor binding data presented here do not resolve these questions in a definitive manner, they do indicate that p32 binds specifically to the signal sequence region of mitochondrial precursor proteins. These data are, therefore, consistent with our previous proposal that p32 functions as a mitochondrial import receptor.

MATERIALS AND METHODS

Materials. Enzymes for molecular biological experiments were from Boehringer Mannheim and New England Biolabs; GeneAmp PCR reagents were from Perkin-Elmer/Cetus; [35S]methionine (1140 Ci/mmol; 1 Ci = 37 GBq) was from NEN; protein A-Sepharose CL-4B was from Pharmacia; sarkosyl (sodium N-lauroylsarcosine) was from Fluka.

Construction of Plasmids. The T7 polymerase expression vector pET-3a (14) was used to express MIRI (p32) and PUT2 genes in E. coli. For the expression of MIRI gene, two restriction sites were introduced in the MIRI gene: an Nde I site at the initiation codon ATG of the MIRI coding sequence and a BamHI site at the 3′ untranslated region. The sense oligonucleotide 5′-CTCCATATGTCGTGGTCGTGC-TCC-3′ and the antisense oligonucleotide 5′-GGCGATCC-

Abbreviations: DTT, dithiothreitol; BSA, bovine serum albumin; RER, rough endoplasmic reticulum.

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TAGTGATTGAATAGAGT-3') were synthesized and the PCR was performed with 1 ng of HindIII digests of pH M77 (pellets were resuspended in 20 mM Tris-HCl, pH 8.3 M urea/Trasylol [50 units/ml]) and subcloned into the Sma I site of pBluescript II SK(-) (Stratagene). The insert containing the MIRI gene was excised with Nde I and BamHI restriction enzymes and subcloned into the T7 expression vector pET-3a, which had been digested with Nde I and BamHI enzymes. The plasmid obtained was designated pNYHM131.

For the expression of the PUT2 gene, the gene was excised from pSP64-Put2 (15) by HindIII restriction enzyme and subcloned into the HindIII site of pBluescript II SK(+), (Stratagene) under control of the T7 promoter. Oligonucleotide-directed mutagenesis (16) was carried out on this plasmid by using the oligonucleotide 5'-ATTCCAGAGA-CATATGCTATCA-3'^1^ to generate an Nde I site at the initiation codon of the PUT2 gene. The Nde I-BamHI fragment containing PUT2 gene was excised from the obtained clone and was subcloned into pET-3a, which had been digested with Nde I and BamHI enzymes. The plasmid obtained was designated pNYHM170.

**Purification of the Expressed Proteins from E. coli.**

BL21(DE3)/pNYHM131 was cultured in 50 ml of LB medium containing ampicillin (0.1 mg/ml) at 37°C (14). When the cell density reached 4 x 10^8 cells/ml, isopropyl-β-D-thiogalactoside and additional ampicillin were added to 1 mM and 0.1 mg/ml, respectively, and the cells were cultured for another 3 hr at 37°C. The cells were collected and washed with 20 mM Tris-HCl (pH 7.5), suspended in 2 ml of buffer A (20 mM Tris-HCl, pH 7.5/5 mM EDTA/1 mM phenylmethylsulfonyl fluoride containing lysozyme (40 μg/ml), and then incubated at 25°C for 15 min. The cells were chilled on ice. The following steps were carried out at 4°C. The cells were disrupted by sonication (five 30-sec bursts at maximum setting) and the lysates were centrifuged at 8,000 x g for 10 min. The pellets were suspended in 1 ml of buffer A, followed by another round of sonication and centrifugation as above. The pellets were suspended in 0.4 ml of buffer A and the proteins were solubilized by adding 20 vol of buffer B [50 mM Tris-HCl, pH 7.5/350 mM NaCl/5 mM EDTA/sarkosyl/Trasylol (50 units/ml)] and then incubated at 25°C for 15 min. The cells were chilled on ice. The translation of protein was determined by SDS/PAGE with BSA as a standard.

For the preparation of the mitochondrial precursor pPut2, BL21(DE3)/pNYHM170 was cultured in 1 liter of the LB medium under the same conditions as described above. The cells were suspended in 20 ml of buffer A containing 0.5 mM DTT and lysozyme (40 μg/ml) and incubated at 25°C for 20 min. The cells were chilled on ice, then disrupted by sonication as described above, and centrifuged at 20,000 x g for 10 min. The supernatant was collected and incubated at 25°C for 15 min, the extracts were centrifuged at 15,000 x g for 10 min at 25°C and the supernatant was again centrifuged at 350,000 x g for 10 min at 20°C. The latter supernatant contained purified p32 at a concentration of 0.55 mg/ml.

For the preparation of the mitochondrial precursor pPut2, BL21(DE3)/pNYHM170 was cultured in 1 liter of the LB medium under the same conditions as described above. The cells were suspended in 20 ml of buffer A containing 0.5 mM DTT and lysozyme (40 μg/ml) and incubated at 25°C for 20 min. The cells were chilled on ice, then disrupted by sonication as described above, and centrifuged at 20,000 x g for 10 min. The supernatant was collected and incubated at 25°C for 15 min, the extracts were centrifuged at 15,000 x g for 10 min at 25°C and the supernatant was again centrifuged at 350,000 x g for 10 min at 20°C. The latter supernatant contained purified pPut2 at 5.8 mg/ml.

**Cell-Free Protein Synthesis.** The in vitro transcription and translation of pCoxIV-DHFR (1); pPut2 (1, 15), preprolactin (17), and Rubisco pS (18) in the presence of [35S]methionine were as described. For the cell-free transcription and translation system, the MIRI gene was excised by Ksp I and HindIII restriction enzymes from pNYHM78 (9) and subcloned into pBluescript II KS+(+) (Stratagene), which had been digested by the same enzymes, under the promoter of the T7 RNA polymerase. The obtained plasmid (designated as pNYHM94) was linearized by Nsi I restriction enzyme and transfected in vitro with T7 RNA polymerase (18). Translation in the presence of [35S]methionine was as described (1).

**Binding Reactions.** p32 (1.38 μg, 5 μl) was diluted with 100 μl of buffer E [50 mM Tris-HCl, pH 7.5/5 mM EDTA/50 mM KCl/0.1% Triton X-100/0.05% sarkosyl/Trasylol (50 units/ml)] and incubated at 25°C for 10 min. After centrifugation at 15,000 x g for 10 min at room temperature, 100 μl of supernatant was incubated for 30 min at 25°C with 1 μl of translation mixture containing [35S]-labeled precursor proteins (4 μl for Rubisco pS). This mixture was then further incubated at 25°C for 2 hr with 10 μl of either anti-p32 or preimmune serum. Buffer E (150 μl) containing 10 mg of protein A-Sepharose CL-4B (which had been washed with the buffer E) was then added. The samples were incubated for 1 hr at 25°C and then centrifuged at 15,000 x g for 30 sec. The pellets were washed three times with 1 ml of buffer W [50 mM Tris-HCl, pH 7.5/5 mM EDTA/150 mM NaCl/0.05% sarkosyl/Trasylol (50 units/ml) and once with 1 ml of 20 mM Tris-HCl, pH 7.5/5 mM EDTA. The pellets were suspended in 60 μl of 8.3 M urea/1.7% (wt/vol) SDS/83 mM DTT/0.05% bromophenol blue. The suspensions were incubated at 100°C for 1 min and centrifuged at 15,000 x g for 1 min. The supernatants were analyzed by SDS/polyacrylamide gel electrophoresis and fluorography.

For the binding of [35S]-labeled p32 to pPut2, 5 μl of pPut2 (2.9 μg) was diluted with buffer E, processed as described above, and incubated with 1 μl of translation mixture containing [35S]-labeled p32. Subsequent binding and immunoprecipitation reactions were as described above.

For the competitive inhibition of [35S]-labeled pCoxIV-DHFR binding to p32 by pPut2, 5 μl of p32 (1.38 μg) was diluted with 100 μl of buffer E containing the corresponding amounts (as shown in Fig. 3) of bovine serum albumin (BSA) or pPut2 purified from *E. coli.***

**Miscellaneous.** The amount of protein was determined by the BCA protein assay (Pierce) with BSA as a standard.

**RESULTS**

A preprotein, pPut2, that contained a signal sequence for protein import into mitochondria and the mitochondrial import receptor (p32) were expressed in *E. coli.* Both expressed proteins were purified and analyzed by SDS/PAGE and Coomassie blue staining (Fig. 1). By densitometric analysis of the gel (data not shown), the purity of p32 and pPut2 was estimated to be >90% and >70%, respectively.

In the first set of experiments, p32 was incubated with various preproteins that were synthesized in cell-free translation systems in the presence of [35S]methionine. Binding of these [35S]-labeled preproteins to p32 (unlabeled) was assessed by immunoprecipitation with anti-p32 antibodies, or in a control, with preimmune serum, and subsequent analysis of the immunoprecipitates by SDS/PAGE and fluorography (Fig. 2). The *in vitro*-synthesized [35S]methionine-labeled preproteins that were tested in these experiments contained either a "mitochondrial" signal sequence specifying protein translocation from the cytosol into the mitochondrial matrix (pPut2 and pCoxIV-DHFR) or an RER signal sequence specifying protein translocation from the cytosol across the RER (preprolactin) or a "chloroplast" signal sequence specifying protein translocation from the cytosol into the chloroplast stroma (Rubisco pS). Synthesis of the mitochondrial...
preproteins pPut2 and pCoxIV-DHFR in the cell-free system yielded not only the full-length preproteins but also truncated forms presumably due to initiation at downstream codons for methionine (Fig. 2, lanes 1 and 4). These truncated forms, therefore, would lack their N-terminal mitochondrial signal sequence. They cannot be imported into mitochondria (data not shown) presumably because of the requirement of the signal sequence to open the protein-conducting channels in both outer and inner mitochondrial membranes (1, 19–21). If p32 were to function in signal-sequence binding, it should bind only the full-length pPut2 and pCoxIV-DHFR but not their N-terminally truncated forms (Fig. 2, lanes 1 and 4). Indeed the full-length forms bound to p32, whereas the N-terminally truncated forms (lanes 1 and 4), even when present in molar excess relative to the full-length preprotein (lane 4), did not bind to p32 (lanes 2 and 5). Moreover, p32 did not bind to preproteins containing either RER (lane 8) or chloroplast (lane 11) signal sequences. Also, coprecipitation with anti-p32 antibodies resulted from specific binding to p32 rather than from nonspecific aggregation of the preproteins during the assay, as control experiments using preimmune serum (lanes 3, 6, 9, and 12) did not yield coprecipitation. Thus, these data indicate that p32 is able to bind specifically to the signal sequence region of preproteins destined for protein import into mitochondria.

Mitochondrial preproteins should also compete with each other for binding to p32. To test for competition, p32 was incubated with in vitro-synthesized 35S-labeled pCoxIV-DHFR either in the absence or in the presence of increasing concentrations of in vivo-synthesized unlabeled pPut2. Binding of the labeled pCoxIV-DHFR to p32 was again assessed by immunoprecipitation with anti-p32 antibodies, SDS/PAGE analysis of the immunoprecipitates and subsequent fluorography (Fig. 3). Indeed, increasing concentrations of pPut2 inhibited binding of pCoxIV-DHFR to p32 (Fig. 3, lanes 2–5). BSA at a concentration similar to the highest concentration of pPut2 did not compete (lane 1), indicating that the observed competition by pPut2 was specific and not due to nonspecific protein concentration effects.

The binding studies carried out so far (Figs. 2 and 3) were done in the presence of a molar excess of p32 by an estimated 4–6 orders of magnitude with respect to ligand. We also tested binding under the reverse conditions—i.e., at a similarly large molar excess of pPut2 with respect to p32. To this end, unlabeled pPut2 expressed in E. coli was incubated with 35S-labeled p32 that was synthesized in the cell-free system. Binding of pPut2 to p32 was assayed by immunoprecipitation with anti-Put2 antibodies, SDS/PAGE of the immunoprecipitates, and subsequent fluorography (Fig. 4). Indeed, p32 was coprecipitated by anti-Put2 antibodies (lane 2) but not by preimmune serum (lane 3). These data indicated that p32 binds specifically to pPut2.

**DISCUSSION**

The in vitro binding data presented here indicate that the signal sequence region of mitochondrial precursor proteins

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**Fig. 1.** Mitochondrial proteins purified after expression in E. coli. Mitochondrial proteins were expressed in E. coli, purified, separated by SDS/PAGE in a 12% polyacrylamide gel, and stained by Coomassie brilliant blue R-250. Lanes: 1, 1.1 μg of p32; 2, 2.9 μg of pPut2. Migration positions of molecular mass standards (Bio-Rad) are shown on the left in kDa.

**Fig. 2.** Binding of in vitro-synthesized [35S]-methionine-labeled precursor proteins to p32. In vitro-synthesized [35S]-methionine-labeled precursor proteins were incubated with p32 expressed in and purified from E. coli. Precursor-p32 complexes were incubated with anti-p32 (lanes 2, 5, 8, and 11) or preimmune serum (lanes 3, 6, 9, and 12) and protein A-Sepharose precipitates were analyzed by SDS/PAGE in a 10% polyacrylamide gel (lanes 1–3) or in a 12% polyacrylamide gel (lanes 4–12) followed by fluorography. Ten percent of the precursor proteins used in the binding reaction were analyzed in lanes 1, 4, 7, and 10. The precursor proteins examined were pPut2 (lanes 1–3), pCoxIV-DHFR (lanes 4–6), preprolactin (lanes 7–9), and Rubisco pS (lanes 10–12). Bands labeled with an arrowhead correspond to N-terminally truncated translation products due to initiation from downstream methionine codons.

**Fig. 3.** Competition between mitochondrial preproteins for binding to p32. Binding of in vitro-synthesized [35S]-methionine-labeled pCoxIV-DHFR to p32 was carried out as in Fig. 2 in the presence of BSA (lane 1) or the absence or the presence of increasing amounts of pPut2 expressed in and purified from E. coli (lanes 2–5). Immunoprecipitation with anti-p32 serum and analysis by SDS/PAGE in a 12% polyacrylamide gel and subsequent fluorography were as in Fig. 2.
Fig. 4. Binding of in vitro-synthesized [35S]methionine-labeled p32 to pPut2. In vitro-synthesized [35S]methionine-labeled p32 was incubated with pPut2 expressed in and purified from E. coli. pPut2–p32 complexes were incubated with anti-Put2 serum (lane 2) or preimmune serum (lane 3), and protein A-Sepharose precipitates were analyzed by SDS/PAGE in a 12% polyacrylamide gel followed by fluorography. Ten percent of p32 used in the binding reaction was analyzed in lane 1.

binds specifically to the previously identified mitochondrial import receptor (p32). This conclusion is based on the following evidence. (i) Two mitochondrial preproteins that contain functionally and structurally diverse mature regions but have a mitochondrial signal sequence bind to p32. These data suggest that it is the signal sequence rather than the mature region of these preproteins that interacts with p32. (ii) N-terminally truncated forms of the tested mitochondrial preproteins lacking their signal sequence no longer bind to p32, again indicating that the signal sequence region of the mitochondrial preproteins is required for binding to p32. (iii) Preproteins containing RER or chloroplast signal sequences did not bind to p32, indicating that p32 contains a binding site only for a mitochondrial signal sequence. These data are consistent with the observed reactivity of p32 with the signal-sequence-mimetic anti-idiotypic antibodies that in turn led to the proposal that p32 functions as a mitochondrial-signal sequence receptor (1). Nevertheless, we do not know whether mitochondrial signal sequences bind to p32 also in vivo. It is conceivable that the observed in vitro binding to mitochondrial signal sequence is an incidental rather than a physiological property of p32. In either case might p32 be recognized by the signal-sequence-mimetic anti-idiotypic antibodies.

As mentioned above, p32 has also been proposed to function as phosphate translocator that catalyzes P_i–H^+ symport across the inner mitochondrial membrane. This assignment is entirely based on results that were obtained by measuring P_i uptake into reconstituted proteoliposomes (12). Although the reported data are clearly consistent with p32 functioning as phosphate translocator, it should be pointed out that there remain several uncertainties regarding this assignment. (i) Purification to homogeneity of p32 from detergent extracts of mitochondrial membranes under conditions where activity is conserved is difficult (12) and has so far not yet been accomplished. As a result, the protein preparations used for proteoliposome reconstitution, although clearly enriched in p32, are not sufficiently pure to be certain that the observed P_i uptake was catalyzed by p32 and not by other proteins. (ii) One cannot be certain whether the observed P_i uptake into proteoliposomes represents translocation of P_i into the aqueous compartment delimited by the proteoliposomes or represents nonspecific binding of P_i on the surface of the proteoliposomes.

Of course, it is possible that p32 is a bifunctional protein that serves as both import receptor and phosphate translocator. There are by now examples for mitochondrial bifunctional proteins (e.g., see refs. 22 and 23). However, p32's bifunctionality may be spurious if one considers the following scenario. If, as suggested, p32 was a signal sequence binding subunit of a protein-conducting channel, such a channel is likely to consist of several distinct but perhaps related subunits, by analogy—e.g., to the acetylcholine receptor, a ligand-gated heterooligomeric ion-conducting channel. Binding of the mitochondrial signal sequence to p32 would open the protein-conducting channel much like the signal sequence of an exported protein in bacteria opens the protein-conducting channel in the bacterial plasma membrane (20). When opened and not occupied by a translocating chain, these protein-conducting channels are large enough to allow diffusion—e.g., of glutamate or Hepes (20). It is likely that they would also allow diffusion of phosphate. Thus, reconstitution of the signal sequence binding subunit of a protein-conducting channel into proteoliposomes might result in the formation of a homooligomeric channel that is very much like the homooligomeric channel that the ligand binding subunit of the acetylcholine receptor can form (24). A homooligomeric channel made up of signal sequence binding subunits (p32) might occasionally open and close even in the absence of the mitochondrial signal sequence, thereby allowing diffusion into and trapping by proteoliposomes, of externally added P_i. Channel opening and/or closing might be affected by sulfhydryl reagents and/or pH. Such a scenario would readily explain the data that have so far been gathered and interpreted in support of p32 being a phosphate translocator.

It should be noted that a similar double-assignment import receptor (25) and phosphate translocator (26) has been made for p36, an integral membrane protein of the chloroplast envelope. Curiously, very similar uncertainties regarding either of these assignments exist also in this case.

In summary, the observed specific in vitro binding between the signal sequence region of mitochondrial precursor proteins and p32 supports, but does not prove, our previous conclusion that p32 functions as a receptor for mitochondrial signal sequences. With regard to the apparently conflicting proposal that p32 functions as a phosphate translocator, it is fair to state that definitive evidence for this assignment is still lacking (for reasons stated above). If, as suggested, p32 serves as the signal sequence binding subunit of a heterooligomeric protein-conducting channel, then the isolation of such a channel and its electrophysiological characterization (e.g., gated open by the mitochondrial signal sequence) might afford an opportunity in the future to provide more definitive evidence for or against this envisaged function of p32.

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