Relationship between the Peptide-sensitive Channel and the Mitochondrial Outer Membrane Protein Translocation Machinery

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The peptide-sensitive channel (PSC), a cationic channel of the mitochondrial outer membrane, is blocked by synthetic mitochondrial presequences and by nonmitochondrial basic peptides such as dynorphin B(1–13). Both types of peptides are imported into mitochondria. However, the import of dynorphin B(1–13) had to be further characterized since its properties differed from those of the general import pathway used by mitochondrial peptides. Cross-linking experiments with iodinated dynorphin B(1–13) led to the labeling of TOM 40/ISP 42, a component of the protein import machinery of the outer membrane. Accordingly, dynorphin B(1–13) could also be used as a presense to direct the import of a cytosolic protein into the mitochondria. Pretreatment of intact mitochondria by trypsin removed components capable of discriminating between true mitochondrial presequences and other basic peptides active on the PSC. After proteolysis, both types of peptides appeared to cross the outer membrane through the same pathway. Involvement of the PSC in the translocation complex was shown by immunoprecipitation of the PSC activity by anti-ISP 42 antibodies. Taken together, the present data reinforce the hypothesis that the PSC is the pore responsible for the translocation of protein through the outer membrane.

Many proteins have to fully or partially cross biological membranes to reach their final destination and thus achieve their biological function. The physicochemical mechanism of translocation through membranes remains largely unknown although the involvement of water-filled channels has been postulated (for example, see Refs. 1–3). Mitochondria provide an excellent model to study this mechanism as they are the site of an intense protein import activity (4). The outer membrane contains a protein translocation complex that includes different receptors capable of discriminating between different subsets of mitochondrial proteins. Following this recognition step, protein translocation appears to occur through an unique pore, the general insertion pore (GIP)1 (5). The mitochondrial outer membrane contains a voltage-dependent cationic channel of large conductance, the peptide-sensitive channel (PSC) (6, 7). The PSC is blocked by small basic peptides derived from mitochondrial presequences (e.g. pCytOX IV(1–12)Y, a peptide corresponding to the first 12 amino acids of cytochrome c oxidase subunit IV precursor) (8–10). Since the electrophysiological characteristics of the PSC blockade were consistent with the translocation of the blocking peptides through the channel, its involvement in protein import into mitochondria was proposed by Henry et al. (8).

The PSC is also blocked by basic peptides unrelated to mitochondrial physiology, such as dynorphin B(1–13). However, dynorphin B(1–13) failed to import the inhibit of a chimeric protein, cytochrome b6-dihydrofolate reductase (DHFR), into the matrix of yeast mitochondria (11). These experiments could not thus establish any direct correlation between the electrophysiological blockade of the PSC and the inhibition of protein import into mitochondria. In order to determine the function of the PSC, the import into yeast mitochondria of the two peptides pCytOX IV(1–12)Y and dynorphin B(1–13) was investigated in vitro (11). Experiments with iodinated peptides showed that both were imported and subsequently degraded. Two import mechanisms could be distinguished: the mitochondrial sequence was translocated into the matrix in a temperature- and potential-sensitive manner and probably along the general import pathway, whereas dynorphin B(1–13) was imported into the intermembrane space by a process that was neither temperature- nor potential-sensitive. This second pathway is likely to involve the PSC (12), and the aim of this work was to study its relationship with the general import pathway.

EXPERIMENTAL PROCEDURES

Materials—Dynorphin B(1–13) was obtained from Sigma, and pCytOX IV(1–12)Y was from Néosystème (Strasbourg, France). pRIHB (fragment of the retinoic acid-induced heparin-binding protein) was a gift from Dr. D. Raulais (INSERM U 118). The cross-linker sulfo-EGS, a water-soluble reagent derived from ethylene glycol-bis(succinimidylic carbonate) (EGS), was obtained from Pierce. Octyl β-glucoside (octyl-β-D-glucopyranoside) and other reagents were obtained from Sigma.

Rabbit anti-ISP 42 sera were a gift of Pr. W. Neupert (University of Munich). They were raised against peptides corresponding to the N- and C-terminal sequences of the protein, and they were used as a 1:1 mixture. Alternatively, the serum against the C terminus was immunopurified using the corresponding peptide (GLDADGNPLGALPQL) immobilized on Affi-Gel-10 beads (Pierce).

Radiolabeling of Basic Peptides—Peptides, resuspended in PBS (phosphate-buffered saline) at a concentration of 1 m·l, were iodinated with 0.5 mCi Na125I (Amersham, France) using iodogen according to e This work was supported in part by grants from the Institut National de la Santé et de la Recherche Médicale (Contract 92-0506), the Centre National de la Recherche Scientifique (UPR 9071 and UPR 9040), and the Direction des Recherches et Etudes Techniques (Contract 93–105). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: GIP, general insertion pore; PSC, peptide-sensitive channel; EGS, ethylene glycol-bis(succinimidylic carbonate); PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; DHFR, dihydrofolate reductase; PCR, polymerase chain reaction; CCCP, carbonyl cyanide m-chlorophenylhydrazone; BSA, bovine serum albumin; EIA, enzymatic immunoassay; AAC, ATP/ADP carrier.

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manufacturer instructions (Pierce).

Preparation of Mitochondria—The preparation of yeast mitochondria has been described previously (11).

Trypsin Treatment of Mitochondria—Yeast mitochondria (1 mg of protein/ml) were treated with 1 mg/ml of either trypsin or bovine serum albumin (BSA) as a control. Trypsin was inhibited by 1 mM EDTA, and the mitochondria was pelleted at 10,000 × g for 5 min. The pellet was washed several times with an excess of isonicotic buffer before its resuspension at a concentration of 10 mg of protein/ml. The integrity of mitochondria was assessed as described previously (11).

Translocation Assay—Translocation of proteins and peptides was assayed as described previously (11).

Construction of Chimeric Proteins—The protocol followed was derived from that described in Vallette et al. (13). Typically, the complete sequence of mouse DHFR was subcloned to a pGEM 4Z vector (Promega, USA). This vector was primed by two oligonucleotides: ON.D corresponding to the sequence of the first three amino acids of the sequence of the DHFR preceded by the sequence of the last six amino acids of the dynorphin B(1–13), plus a BamHI site (5’-TTT GGATCCATGTACGGTTTC-CAAA TACATGCTAT-3’). The resulting amplification product was cloned into pGEM 4Z to generate pGEM d-DHFR and the DNA encoding the chimera was sequenced. This construct was used to generate RNA and to synthesize the d-DHFR protein in vitro. The N-terminal sequences of these constructs are given in Table I.

Cross-linking Experiments—Yeast mitochondria (100 μg of protein) were incubated in the presence of 125I-labeled peptides for 5 min and sulfo-EGS was added at a concentration of 0.5 mM. The mixture was incubated at 30 °C for 5 min. The reaction was stopped by the addition of 10 mM glycine as described by Font et al. (14). Immunoprecipitation of cross-labeled products was performed as described in Harlow and Lane (15). Briefly, mitochondrial proteins were extracted in PBS supplemented with 1% Triton X-100 and incubated overnight at 4 °C with sera prebound to protein A-Sepharose 4B (see suspension). The suspension was centrifuged, and the pellet was then washed extensively in a buffer containing 300 mM NaCl and either Triton X-100 or Nonidet P-40 (1%). The cross-linked products bound to the antibodies were analyzed by SDS-PAGE and autoradiography.

Solubilization and Reconstitution Procedure—The PSC activity of yeast mitochondria from a porin-less mutant (B5 10A) was reconstituted as described previously for mammalian mitochondria (16) except that 0.75% octyl β-glucoside was used to solubilize yeast mitochondria. An antibody from an anti-Dynorphin B(1–13) raised against peptides derived from ISP 42 sequence (about 2.5 mg of protein) or control sera were adsorbed onto protein A-Sepharose 4B. This mixture was incubated with solubilized fractions (10 μg of proteins) in buffer A (150 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin, 1.25 μg/ml leupeptin, 0.75% octyl β-glucoside, 20 mM Hepes-KOH, pH 7.5) at 4 °C overnight before centrifugation. The beads were then washed once with buffer A and twice with buffer B (50 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin, 2 μg/ml leupeptin, 10 mM Hepes-KOH, pH 7.5). Supernatants from unbound and washing fractions were pooled and diluted in buffer B to reconstitute the PSC activity. The reconstituted pellet was divided into two parts: the first one was assayed by EIA as described by Harlow and Lane (15) for quantification of ISP 42 or of the ATP/ADP carrier (AAC), and the second part was added to liposomes to form proteoliposomes used to quantify the PSC activity.

Electrophysiological Recordings—Proteoliposomes were prepared, and bilayers were formed at the tip of microelectrodes by the tip-dip technique as described by Thieffry et al. (6). The channel content of a given fraction was quantified by the frequency of the characteristic electrophysiological activity (7) and defined as the total number of channels divided by the total number of seals (i.e. seals exhibiting PSC activity plus seals with no electrical activity). Approximately 20 seals were performed on each fraction. Positive seals never contained more than one channel. Control experiments were carried out to verify that, in the range of protein concentrations used, the channel frequency was linearly related to the protein concentration. Comparisons of various fractions were performed at a constant protein concentration in the proteoliposomes formed from immunodepleted or control fractions. Four independent experiments were used in a statistic test (t test) to determine the significance of the difference in the channel frequency observed between the different fractions. Exposure of the channels to peptides was achieved by transferring the tip of the pipette into a bath containing peptides.

RESULTS

Cross-linking of Dynorphin B(1–13) with Yeast Mitochondrial Membranes—Porin-deficient yeast mitochondria were incubated with 1 μM 125I-labeled dynorphin B(1–13) for 5 min at 30 °C under conditions that allowed its import into the organelle (11). The bifunctional reagent sulfo-EGS was used to reticulate the proteins interacting with the peptide. Several proteins were labeled, but only two were reproducibly cross-linked to the iodinated peptide (Fig. 1). These two bands migrated with apparent molecular weights of 44 and 32 kDa. Assuming that one molecule of peptide was bound to one molecule of protein by the cross-linking agent (molecular mass of the peptide plus the cross-linker is 2 kDa), their molecular masses would be 42 and 30 kDa. The specificity of this labeling was assessed by competition with a 50-fold excess of different unlabeled peptides. An excess of dynorphin B(1–13) strongly inhibited the labeling of the 44-kDa band and reduced, to a much lesser extent, that of the 32-kDa band (data not shown). The addition of an excess of pRhB (see Table I), a peptide inactive on the PSC and dynorphin B(1–13) import, had no affect on the labeling (data not shown). Finally, an excess of pCytoX IV(1–12)Y, which inhibited the in vitro import of dynorphin B(1–13) (11), drastically decreased the labeling of the 44-kDa band and had a weaker effect on that of the 32-kDa band (Fig. 1). In addition, a mild trypsin treatment of the labeled membranes decreased the radioactive 44-kDa band without affecting the 30-kDa band (data not shown). These results showed that dynorphin B(1–13) could be cross-linked to a protein of 42 kDa of the outer membrane and that this binding was inhibited by pCytoX IV(1–12)Y.

Since the major protein of the translocation machinery of the outer membrane is ISP 42/TOM 40 (17, 18), we examined whether it might correspond to the 44-kDa labeled band. We thus used antibodies raised against synthetic peptides derived either from the N- or C-terminal sequences of ISP 42. Yeast mitochondria were cross-linked with 10 μM 125I-labeled dynorphin B(1–13) in order to increase the labeling of the 44-kDa band. Under these conditions, additional bands were observed, including a component of 52 kDa (Fig. 2, lane 1). This 52-kDa band was present in some but not all experiments performed at 1 μM protein concentration, and it was not observed further. The 51 proteins were then extracted in PBS containing 1% Triton X-100. The solubilized fraction, which contained most of the radioactivity, was immunoprecipitated overnight at 4 °C in the presence of either anti-ISP 42 antisera or a nonimmune serum, immobilized on protein A-Sepharose 4B. The beads were washed with increasing concentrations of salt (up to 300 mM NaCl) and/or 1% Nonidet P-40 in order to prevent immu-
The chimeric protein d-DHFR was, therefore, unlikely to interact with the PSC (8, 11).

The [35S]methionine-labeled chimeric proteins, d-DHFR and dΔ(1–7)-DHFR, were incubated in vitro with yeast mitochondria in the import buffer for 20 min at 30 °C. The mitochondria were then pelleted at 10,000 \times g for 10 min. The pellet and the supernatant were analyzed by SDS-PAGE and autoradiography. The chimera protein dΔ(1–7)-DHFR was exclusively found in the supernatant (Fig. 3A). On the other hand, d-DHFR was totally associated with the pellet. An important fraction of the bound radiolabeled protein was resistant to trypsin, indicating that it was imported into the mitochondria (Fig. 3B).

This result showed that dynorphin B(1–13) can direct a cytosolic protein into the mitochondria, thus behaving like a mitochondrial presequence.

We studied the effect of basic peptides on the import of d-DHFR. pRIHB, which is not active on the PSC, did not block the import, whereas both pCytOX IV(1–12)Y and dynorphin B(1–13) did (Fig. 4A). As previously noted (11), neither pRIHB nor dynorphin B(1–13) had any effect on import of the first 167 amino acids of yeast cytochrome b2 fused to mouse dihydrofolate reductase (Fig. 4B). It should be noted that carbonyl cyanide m-chlorophenyl hydrazone (CCCP), which dissipates the inner membrane potential, blocks the import but not the binding of the two chimeric proteins (Fig. 4, A and B).

These results extend previous observations (11). At 30 °C, the translocation of natural presequences (pCytOX IV(1–12)Y) or presequence-driven cytosolic protein (b2-DHFR) is inhibited by natural presequences and unaffected by dynorphin B(1–13). On the contrary, the import of dynorphin B(1–13) and that of the chimera d-DHFR are inhibited by both natural presequences and dynorphin B(1–13).

**Trypsin-sensitive Components of the Outer Membrane Discriminate between Dynorphin B(1–13) and the Synthetic Mitochondrial Presequence pCytOX IV(1–12)Y**—A possible explanation for the difference observed between the import of natural presequences and that of dynorphin B(1–13) is the presence of receptors on the outer membrane surface (11). These receptors would discriminate between the two types of peptides that would then be transported along the same pathway. To test this hypothesis, isolated yeast mitochondria were subjected to mild proteolysis, as described earlier (11). Isolated yeast mitochondria were incubated at 0 °C with trypsin or BSA (as mock treatment) in a 1:1 ratio for 10 min. At the end of the incubation, mitochondria were washed extensively in an isotonic buffer containing 10 mg/ml soybean trypsin inhibitor and were finally resuspended in an import buffer at a concentration of 1 mg/ml of protein. The outer membrane was not disrupted since the activity of an intermembrane space enzyme, adenylate

**FIG. 1. Cross-linking of dynorphin B(1–13) with mitochondria.** Cross-linking experiments were carried out by incubation, for 10 min at 30 °C, of yeast mitochondria (100 μg of protein) with 1 μM radiolabeled dynorphin B(1–13), 1 mM final sulfo-eosin (lanes 1 and 3), and in the absence (lanes 1 and 2) or in the presence (lane 3) of 50 μM pCytOX IV(1–12)Y. The reactions were terminated by the addition of 20 mM glycine. The mitochondria were isolated by centrifugation and then subjected to analysis by 13% SDS-PAGE and autoradiography. The position of the major labeled bands is indicated by arrows.

**FIG. 2. Interaction of dynorphin B(1–13) with ISP 42.** Cross-linking using yeast mitochondria (100 μg of protein) and 10 μM 125I-labeled dynorphin B(1–13) was performed as described in Fig. 1. At the end of the reaction, the samples were subjected to immunoprecipitation except for lane 1, which contained 100 μg of cross-linked mitochondrial proteins as a control. The antibodies used were either a non-immune rabbit serum (Lane 2) or a polyclonal serum raised against synthetic peptides derived from N- and C-terminal sequences of ISP 42 (lane 3).
kinase, was not decreased during the treatment and the washing procedure (data not shown). The in vitro import of the preprotein b2-DHFR was almost completely abolished in trypsin-treated mitochondria (data not shown). However, this treatment did not affect the conductance of the PSC and did not modify its sensitivity to basic peptides. It also did not produce any detectable change of ISP 42 in immunoblotting experiments (data not shown).

The import of the presequence 125I-labeled pCytOX IV(1–12)Y was analyzed in trypsin and in mock-treated mitochondria. When labeled peptides were incubated with mitochondria, peptides were degraded upon import into the matrix (11). Thus, the degradation of the presequence by metallopeptidases present in the mitochondrial matrix was used to monitor the import (11, 12). When mitochondria were pretreated with trypsin, peptide import was less affected than that of proteins. pCytOX IV(1–12)Y (1 µM) degradation remained a linear function of time, but it was completed within 60 min, whereas completion required only 30 min in mock-treated mitochondria (data not shown). A similar effect of trypsin pretreatment was observed on the import of 125I-labeled dynorphin B(1–13). Addition of the uncoupler CCCP inhibited import of 125I-labeled pCytOX IV(1–12)Y in trypsin-treated mitochondria (Fig. 5), as previously shown in intact mitochondria (11).

In agreement with previous results (11), the import of 125I-labeled pCytOX IV(1–12)Y in untreated mitochondria was inhibited in the presence of an excess of cold pCytOX IV(1–12)Y, and it was not affected by an inactive peptide, pRIHB (Fig. 5). This import was not inhibited by dynorphin B(1–13) although this peptide is active on PSC (Fig. 5). To the contrary, in trypsin-treated mitochondria, this discrimination was abolished since the import of 125I-labeled pCytOX IV(1–12)Y was inhibited by an excess of both pCytOX IV(1–12)Y and dynorphin B(1–13) (Fig. 5). The import remained unaffected by pRIHB.

Mitochondria Can Be Depleted of PSC Activity by Immunoprecipitation with Anti-ISP 42 Antibodies—The existence of a common import pathway for mitochondrial and nonmitochondrial peptides active on the PSC justified more investment in order to determine the precise relationship between PSC and GIP, of which ISP 42 is the main component (19). For this purpose, we tested the ability of anti-ISP 42 antibodies to deplete the PSC activity.

Fe`vre et al. (16) have shown that bovine PSC can be solubilized and reconstituted in an active form by a method derived from that published by Yu et al. (20) for the solubilization of functional rough endoplasmic reticulum. We used a similar method to solubilize and reconstitute the yeast PSC from a porin-less mutant strain. The mitochondrial proteins were progressively solubilized in 0.75% octyl β-glucoside. After slow dilution in a detergent-free buffer, the solubilized material was centrifuged, and the PSC activity present in the reconstituted pellet was estimated by the tip-dip technique after incorporation into proteoliposomes (6). The reconstituted channels exhibited an electrical activity identical to that of the native yeast PSC based on the following criteria: (i) they had the same conductance levels and the same voltage-dependence (Fig. 6, left); (ii) they were sensitive to pCytOX IV(1–12)Y, which induced a characteristic voltage-dependent blockade (Fig. 6, right), and to dynorphin B(1–13) (data not shown). It should be noted that, under the conditions used for solubilization and...
with the porin-less mutant strain, we observed no other electrical activity. On the contrary, increasing the concentration of octyl β-glucoside (to 1% and above) resulted in a decrease in the number of active, well defined PSC and led to the appearance of ill defined conductances (data not shown). A similar result has been reported for bovine PSC (16).

In order to deplete the membrane in ISP 42, the fraction solubilized by 0.75% octyl β-glucoside and containing the PSC was incubated with a suspension of anti-ISP 42 IgG immobilized on protein A-Sepharose 4B. Various nonimmune sera and sera directed against nonrelevant mitochondrial proteins (mitochondrial porin, AAC, and cytochrome c oxidase) were used as a control for nonspecific immunoprecipitation. Proteins were allowed to bind to the IgG-protein A-Sepharose complex during overnight incubation at 4 °C. After this incubation, the mixture was centrifuged at 10,000 × g for 10 min at 4 °C and the supernatant was kept at 4 °C. The pellet was washed once with the solubilization buffer (containing 0.75% octyl β-glucoside) and twice with a detergent-free buffer containing 50 mM NaCl. Supernatants from the different washings were pooled and added to the unprecipitated protein fraction. These fractions were slowly diluted as described by Févre et al. (16) over a 2-h period at 4 °C and then centrifuged at 130,000 × g for 3 h at 4 °C. The pellets were resuspended in the low ionic buffer and separated in two aliquots. The first was used to determine the amount of unprecipitated ISP 42, and the second one was incorporated into proteoliposomes to assay the PSC activity. No difference in the frequency of observation of PSC was observed between untreated fractions and fractions treated with nonrelevant sera (data not shown).

The amount of ISP 42 was estimated by EIA (15) in fractions treated by anti-ISP 42 antibodies and by control IgG (Fig. 7A). The specificity of the precipitation was controlled by assaying the amount of AAC by the same technique. The relative abundance of the PSC in immunodepleted and control fractions (Fig. 7B) was quantified by measuring the frequency of observed characteristic electrical activity in tip-dip bilayers (7). Under these conditions, anti-ISP 42 depleted the samples of 75% of the ISP 42 (Fig. 7A) with a minimal effect on the AAC. The PSC activity was decreased to a similar degree in the anti-ISP 42-depleted fractions (Fig. 7B).

We also used affinity purified anti-ISP 42 IgG to ascertain the specificity of this immunodepletion. In this experiment, the purified antibody was neutralized by addition of an excess of the peptide used to raise the antiserum. No or little immunoprecipitation was observed under these conditions. Immunoprecipitation performed in the presence of the irrelevant pep-

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**FIG. 5.** Effect of a proteolytic pretreatment of the mitochondria on the inhibition of pCytOX IV(1–12)Y import by peptides. Yeast mitochondria (1 mg of protein) were pretreated with either 1 mg of BSA or trypsin for 10 min at 0 °C. After inhibition and extensive washing, the mitochondrial pellet was resuspended in the import competent buffer at a concentration of 1 mg/ml. Control and trypsin-treated mitochondria were incubated for 60 min at 30 °C in the presence of 1 μM 125I-labeled pCytOX IV(1–12)Y and of 100 μM cold peptides, as indicated. Peptides were analyzed by SDS-PAGE. The presequence was degraded in the mitochondria, and therefore, undegraded labeled material is essentially non-imported.

**FIG. 6.** Electrical properties of reconstituted yeast PSC. Yeast mitochondria were solubilized by addition of octyl β-glucoside to 0.75% final concentration and centrifugation. The supernatant was reconstituted into proteoliposomes by dilution of the detergent, centrifugation, and freezing and thawing of the pellet in the presence of liposomes. The electrical activity of the proteoliposomes was tested by the tip-dip technique. It was recorded at different potentials, indicated at the left of the figure, before exposure of the channel to 20 μM pCytOX IV(1–12)Y (Control). The reconstituted channel properties were similar to those of the native channel. After transfer of the pipette tip to a bath containing the peptide, a voltage-dependent blockade similar to that observed in native channels, developed immediately (right side). It was characterized by brief closures, the frequency of which increased when the voltage was decreased below 50 mV. The voltages indicated at the left of the traces correspond to Vpipette − Vbath. The open state is indicated by a dotted line. The three main conductance levels (3, 2, 1, in order of decreasing conductance), separated by jumps of 330 pS, are shown at the right of the traces recorded at −40 mV. Closures appear as downward deflections at positive potentials and as upward deflections at negative potentials. The signal was filtered at 2.5 kHz and sampled at 5 kHz.
obtained with the neutralized antibody. The proteoliposomes used were prepared from the same control and ISP 42 depleted fractions. The proteoliposomes were analyzed for their contents in ISP 42 by EIA. In each experiment, the amount of ISP 42 in the immunodepleted fraction was compared with that in the control fraction, which was taken as 100%. Results are means ± S.E. of four different experiments. B, the channel frequency was estimated by the tip-dip technique. The proteoliposomes used were prepared from the same control and ISP 42 depleted fractions as the ones used in A. They had the same protein/lipid ratio. Results are expressed as the means ± S.E. of the PSC frequency in four different experiments (same as in A). They are significantly different (p < 0.025), using a t test with four paired observations.

tide pRIHB decreased ISP 42 content and PSC frequency by 50 and 55% (n = 2), respectively, as compared with the results obtained with the neutralized antibody.

DISCUSSION

Peptide Dynorphin B(1–13) Is Imported into Mitochondria Along the Protein Import Pathway—Recently, Gaikwad and Cumsky (21) have shown in cross-linking experiments that pCytOX IV(1–22), a peptide derived from the presequence of cytochrome c oxidase subunit IV, interacted with several outer membrane proteins including two proteins of 42 and 70 kDa. These two proteins were, respectively, identified in immunoprecipitation experiments as TOM 40/ISP 42, the main component of the general insertion pore of the outer membrane, and TOM 70/Mas 70p, one of the outer membrane surface receptors for protein. This study demonstrated that this experimental approach could identify mitochondrial components of the import machinery. We have followed this approach using dynorphin B(1–13) in place of a natural presequence in order to identify the corresponding import machinery. We used experimental conditions in which most of dynorphin B(1–13) was associated with mitochondria but was not completely imported (11). A protein of 42 kDa was reproducibly labeled by dynorphin B(1–13). This labeling was inhibited under conditions that prevented translocation of dynorphin B(1–13) through the outer membrane, i.e., in the presence of an excess of pCytOX IV(1–12)/Y but not of pRIHB (a control peptide that has no effect on this import or on the PSC activity). These results clearly indicated that the interaction of the 42-kDa protein with dynorphin B(1–13) could be correlated with translocation of the peptide across the outer membrane. Immunoprecipitation experiments identified the 42-kDa band as ISP 42 (Fig. 1B). We used immunoprecipitation conditions (in the presence of Triton X-100 or Nonidet P-40 and 300 mM NaCl) that do not allow the coprecipitation of other components of the translocation complex (22). It is therefore unlikely that the peptide cross-linked with a 42-kDa protein coprecipitating with ISP 42.

We also observed an interaction between dynorphin B(1–13) and a protein of 30 kDa. Gaikwad and Cumsky (21) showed that a component of a similar molecular mass was also labeled in yeast mitochondria by pCytOX IV(1–22). According to these authors, this protein is different from the AAC and from the p32 protein, a putative receptor protein (23, 24). In bovine adrenal cortex mitochondria, pCytOX IV(1–12)Y has also been shown to label a protein of a similar molecular mass, but it was localized in the inner membrane (14).

The present study confirms that of Gaikwad and Cumsky (21) and demonstrates that peptides can be used to identify components of the import machinery such as a receptor (TOM 70) or the general insertion protein TOM 40 by cross-linking. Originally, TOM 40/ISP 42 had been identified by photolabeling using a precursor protein blocked during its translocation (25). Precursors bind to the translocation complex on the cis (cytosolic) side and possibly on the trans side, in the intermembrane space (26, 27). Cross-linking might involve peptides still bound to the cis side but not to the trans side since the cross-linked material is sensitive to trypsin. Alternatively, cross-linking might occur during the translocation through the pore. Analysis of the block of PSC by peptides derived from pCytOX IV showed that the closures attributed to the translocation of the peptide had a maximal duration of the order of 1 ms around 0 mV (10), which is assumed to be the physiological situation.

Dynorphin B(1–13) Acts as Leader Sequence for Translocation into the Mitochondria—A number of basic peptides that share some biophysical properties with mitochondrial presequences have been shown to induce translocation of cytosolic proteins into mitochondria when fused to their N-terminal region (28). Because the import of dynorphin B(1–13) does not share all the characteristics of that of presequences (11), we have tested the ability of this peptide to act as a leader sequence. We have thus constructed a protein in which it precedes DHFR, a cytosolic protein. We have shown that this protein (d-DHFR) is imported into yeast mitochondria as indicated by its association with a mitochondrial pellet and resistance to proteolysis. This import was inhibited by the uncoupling agent CCCP. In contrast, a shorter peptide lacking the first seven residues of dynorphin B(1–13), among which are positively charged amino acids, did not drive the import of the chimera dΔ(1–7)-DHFR. In addition, the import of d-DHFR was inhibited by basic peptides active on the PSC. In contrast to what was observed for b5-DHFR, this import was inhibited by both pCytOX IV(1–12)/Y and dynorphin B(1–13). This difference indicates that the pathway responsible for the import of d-DHFR and dynorphin B(1–13) shares some elements (such as TOM 40/ISP 42) with the pathway responsible for the translocation of natural presequences but that the two pathways differ, presumably at the level of receptors.

Dynorphin B(1–13) and pCytOX IV(1–12)/Y Are Imported through the Same Pore in the Outer Membrane—125I-Labeled dynorphin B(1–13) import and dynorphin B(1–13) driven protein import were inhibited by both dynorphin B(1–13) and the presequence, whereas 125I-labeled pCytOX IV(1–12)/Y import and precursor import were only sensitive to presequence. This difference might be explained by the interaction of the peptides.
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with different surface receptors prior to their translocation to a second site as suggested by Mayer et al. (26). Alternatively, a unique surface receptor binding the peptides with different affinities might be proposed. However, the latter hypothesis is unlikely since the imports of the two types of peptides had different sensitivities to temperature and trypsin treatment (11). In order to explore the events following the recognition/binding phase, mitochondria were treated with trypsin under conditions that did not disrupt the outer membrane. Since this treatment had a less severe effect on peptide import than on precursor import, trypsinized mitochondria were tested for 

125I-labeled pCytOX IV(1–12)Y import. We have shown that this treatment suppressed the discrimination between dynorphin B(1–13) and pCytOX IV(1–12)Y since both peptides were then able to inhibit the translocation of the presequence into the mitochondria (Fig. 5). This result indicates that, in trypsin-treated mitochondria, the import of pCytOX IV(1–12)Y and dynorphin B(1–13) were competitive and took place through the same pore. This unique peptide pathway appears to be identical to the protein translocation pathway as the import of pCytOX IV(1–12)Y in trypsin-treated mitochondria remained dependent upon the mitochondrial transmembrane potential (Fig. 5).

Protease-sensitive receptors specifically bind mitochondrial presequences prior to their translocation into the mitochondria through a protease-resistant site that includes TOM40/ISP 42 (19). The binding and recognition of mitochondrial proteins appeared to require several different receptors with complex recognition patterns (5, 29). Several of these receptors have been identified in Saccharomyces cerevisiae, including TOM 20/Mas 20p and TOM 22/Mas 22p, which appear to bind basic and amphiphilic mitochondrial presequences, and TOM 70/Mas 70p, which supports AAC import (19, 29). As dynorphin B(1–13) inhibits neither the import of a preprotein (11) nor that of AAC, the nature of the receptor/bindings sites involved in the recognition of dynorphin B(1–13) remains to be elucidated.

The PSC Is Likely to Be the Protein Import Pore—The PSC was originally described as a large cationic pore (6), but the analysis of its interaction with basic peptides suggested that it might be permeable to such peptides, and it was hypothesized to be involved in protein translocation (8–10, 30). We show in this work that dynorphin B(1–13) is imported along the protein import pathway as illustrated by the cross-linking experiments. Accordingly, the fact that all peptides active on PSC are translocated through the outer membrane, alone or as a presequence, suggests strongly that this channel might be related to the putative translocation pore. However, the possibility remained that PSC and GIP were distinct pores: the blockade of PSC could result from an interaction of the peptides with the gating mechanism, whereas translocation would occur only through the GIP. It was thus necessary to precisely the structural relationship between PSC and GIP. For this purpose, membrane proteins were solubilized, the supernatant was immunoprecipitated by anti-ISP 42 antibodies, and the PSC activity was assayed after reconstitution. Immunoprecipitation of the detergent extract resulted in a decrease of PSC activity in the reconstituted fraction. Although immunodepletion was not complete in our conditions, we observed a quantitative corre-

lation between the amount of precipitated ISP 42, as measured by ELISA, and the decrease of PSC activity, using either an immunosorbent prepared with total IgG from an anti-ISP 42 serum or with immunopurified anti-ISP 42 antibodies. The specificity of immunodepletion was established by the fact that it was inhibited by the peptide used to raise the antiserum. However, this observation does not indicate that ISP 42 is the only component of the PSC. Octyl β-glucoside might preserve the integrity of the translocation complex, at least at low concentrations. When higher concentrations of the detergent were used, the appearance of various ill-defined conductances might reflect disorganization of this complex.

The present report strengthens the idea that PSC is the protein conducting pore of the outer membrane translocation complex. The translocation machinery of the outer membrane would thus result from the dynamic assembly of a multimeric protein complex around a cationic channel.

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REFERENCES

1. Singer, S. J., and Yaffe, M. P. (1990) Trends. Biochem. Sci. 15, 369–373
2. Simon, S. M., and Blobel, G. (1991) Cell 65, 371–380
3. Simon, S. M., Peskin, C. S., and Oster, G. F. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 3770–3774
4. Attardi, G., and Schatz, G. (1988) Annu. Rev. Cell Biol. 289–333
5. Lill, R., and Neupert, W. (1996) Trends Cell Biol. 6, 56–61
6. Thieffry, M., Juin, P., Goldschmidt, D., and Henry, J. P. (1988) EMBO J. 7, 1449–1454
7. Chich, J. F., Goldschmidt, D., Henry, J. P., and Thieffry, M. (1991) Eur. J. Biochem. 196, 29–35
8. Henry, J. P., Chich, J. F., Goldschmidt, D., and Thieffry, M. (1989) J. Membr. Biol. 112, 139–147
9. Chich, J. F., Neyton, J., Pellecchi, M., Fevre, F., and Henry, J. P. (1992) Biophys. J. 63, 333–339
10. Fevre, F., Henry, J. P., and Thieffry, M. (1994) Biophys. J. 66, 1887–1894
11. Vallette, F. M., Juin, P., Pellecchi, M., and Henry, J. P. (1994) J. Biol. Chem. 269, 13367–13374
12. Juin, P., Pellecchi, M., Sagne, C., Henry, J. P., Thieffry, M., and Vallette, F. M. (1995) Biochem. Biophys. Res. Commun. 211, 92–99
13. Vallette, F., Mege, E., Reiss, A., and Adesnik, M. (1989) Nucleic Acids Res. 17, 723–733
14. Fort, E., Goldschmidt, D., Chich, J. F., Thieffry, M., Henry, J. P., and Gautheron, D. C. (1991) FEBS Lett. 279, 105–109
15. Harlow, E., and Lane, D. (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
16. Fevre, F., Thieffry, M., and Henry, J. P. (1993) J. Bioenerg. Biomembr. 25, 55–60
17. Baker, K. P., Schaniel, A., Vestweber, D., and Schatz, G. (1990) Nature 348, 605–609
18. Baker, K. P., and Schatz, G. (1996) Nature 349, 205–208
19. Kiebler, M., Becker, K., Pfanner, N., and Neupert, W. (1993) J. Membr. Biol. 135, 191–207
20. Yu, Y., Zhang, Y., Sabatini, D. D., and Kreibich, G. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 9931–9935
21. Glick, A. S., and Cumsky, M. G. (1994) J. Biol. Chem. 269, 6437–6443
22. Kiebler, M., Pfanner, H., Sollier, T., Griffiths, G., Hartmann, H., Pfanner, N., and Neupert, W. (1990) Nature 348, 610–616
23. Pain, D., Murakami, H., and Blobel, G. (1990) Nature 347, 444–448
24. Pfanner, H., Pfanner, N., and Neupert, W. (1989) J. Biol. Chem. 264, 34–39
25. Vestweber, D., Brunnier, J., Baker, A., and Schatz, G. (1989) Nature 341, 205
26. Mayer, A., Neupert, W., and Lill, R. (1995) Cell 80, 127–137
27. Bolhger, L., Junne, T., Schatz, G., and Lithgow, T. (1995) EMBO J. 14, 6318–6320
28. Allison, D. S., and Schatz, G. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 9011–9015
29. Lithgow, T., Glick, B. S., and Schatz, G. (1995) Trends Biochem. Sci. 20, 98–101
30. Henry, J. P., Juin, P., Vallette, F., and Thieffry, M. (1996) J. Bioenerg. Biomembr. 28, 101–108