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The Carboxyl-terminal Two-thirds of the ADP/ATP Carrier Polypeptide Contains Sufficient Information to Direct Translocation into Mitochondria*

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The precursor of the mitochondrial inner membrane protein ADP/ATP carrier is cytoplasmically synthesized without an amino-terminal peptide extension. We constructed a truncated precursor lacking the 103 amino acids from the amino terminus (about a third of the protein). Import of the truncated precursor into mitochondria showed the import characteristics of the authentic precursor, including nucleoside triphosphate dependence, requirement for a protease-sensitive component on the mitochondrial surface, two-step specific binding to the outer membrane, and membrane potential-dependent translocation into the inner membrane. We conclude that, in contrast to all other mitochondrial precursor proteins studied so far, domains of the ADP/ATP carrier distant from the amino terminus can carry specific targeting information for transport into mitochondria.

The transport of proteins into mitochondria involves several distinct steps: synthesis of precursors on cytosolic polysomes, nucleoside triphosphate (NTP)-dependent unfolding of precursors, binding of precursors to receptor sites on the mitochondrial surface, insertion into the outer membrane, translocation via contact sites between outer and inner membranes, requirement for a membrane potential ($\Delta\psi$) for insertion into the inner membrane, and proteolytic removal of amino-terminal peptide extensions (presequences) (1, 2).

There is abundant evidence that presequences contain sufficient information for targeting of proteins to mitochondria (reviewed in Ref. 3). Precursors from which the presequences have been removed are usually not imported (3, 4). The question arises, where is the targeting information contained in precursors which are synthesized without cleavable presequences? Studies with the 70-kDa protein of the yeast outer mitochondrial membrane (5) and the ADP/ATP carrier of the yeast inner mitochondrial membrane (6) suggest that the targeting information is contained in the amino-terminal part of the protein. Mutations in the extreme amino terminus of F_1-ATPase subunit β allowed import of subunit β without a presequence (4). Thus, it was generally assumed that amino-terminal protein sequences carry the mitochondrial targeting signals.

In order to study whether the amino-terminal part of the *Neurospora crassa* precursor to the ADP/ATP carrier is essential for targeting, we constructed a precursor lacking the amino-terminal 103 amino acid residues. Surprisingly, this truncated precursor ($C^{104-313}$)[1] was imported into mitochondria. Its import showed the import properties of the authentic ADP/ATP carrier including a requirement for NTPs, for a protease-sensitive component on the mitochondrial surface, and for $\Delta\psi$ (7–12). The binding to the outer membrane could also be resolved into two distinct steps (11, 12). We conclude from these observations that the carboxyl-terminal two-thirds of the ADP/ATP carrier contain specific mitochondrial targeting information.

EXPERIMENTAL PROCEDURES

DNA Manipulations and Construction of $C^{104-313}$—A full-length cDNA coding for the ADP/ATP carrier (13) was isolated from a *N. crassa* library (14). Cloning, shortening of the 5′ end of the cDNA with Bal31 (Biolabs), ligation into pGEM 4 (Promega), and transformation into *Escherichia coli* strain DH1 were carried out essentially as described (15, 16). One clone (KPH6) gave rise to a translation product of 22 kDa (recognized by an antiserum prepared against isolated ADP/ATP carrier) when the truncated cDNA was transcribed with SP6 RNA polymerase and the capped transcripts were translated in a reticulocyte lysate. DNA sequencing (17) of both strands with an SP6 primer and a primer (5′GGCGGACTTGGCGTCGTTGG3′) corresponding to nucleotide positions 459–440 of the antisense strand of ADP/ATP carrier cDNA revealed that the cDNA had been truncated as result of the Bal31 treatment to nucleotide position 227 (13). The predicted molecular weight of a protein starting at the next available ATG (nucleotide position 310) is 22,313. This correlates very well with the observed apparent molecular mass of 22 kDa. This implies that the first 103 amino acid residues of the protein are deleted.

Import of $C^{104-313}$ into Neurospora Mitochondria—Isolation of *Neurospora* mitochondria and binding and import of radiolabeled precursor were performed as described previously (9, 11, 12, 18). The reaction volume for binding and import was 200 μl including a buffer containing 3% (w/v) BSA (9). Each reaction contained 50 μg of mitochondrial protein.

RESULTS

Import of $C^{104-313}$ into Mitochondria Requires $\Delta\psi$ and NTPs—A truncated precursor (M_r 22,313) of the ADP/ATP carrier lacking the amino-terminal 103 amino acid residues was constructed as described under "Experimental Procedures." Reticulocyte lysate containing the truncated precursor ($C^{104-313}$) was incubated with isolated *Neurospora* mitochondria in the presence of ascorbate plus TMPD which generates a membrane potential via complex IV of the respiratory chain (19). After incubation at 25 °C for 25 min, proteinase K was added at a concentration of 250 μg/ml (Fig. 1, Reaction 1). In Reactions 2 and 3 of Fig. 1, $\Delta\psi$ was dissipated by the K^+ ionophore valinomycin (in the presence of 80 mM K^+ outside), the inhibitor of complex III antimycin A, and the inhibitor of the F_oF_1-ATPase oligomycin. In the presence of $\Delta\psi$, $C^{104-313}$ was imported into a protease-protected location (Fig. 1, Reaction 1), whereas in the absence of $\Delta\psi$, import was strongly

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[1] The abbreviations used are: $C^{104-313}$, carboxyl-terminal two-thirds of the ADP/ATP carrier; BSA, bovine serum albumin; TMPD, *N,N,N′,N′*-tetramethylphenylenediamine.

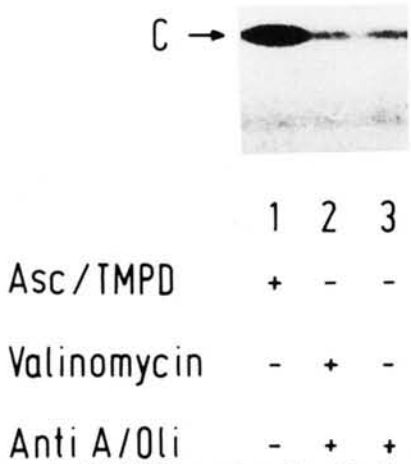

FIG. 1. **Import of $C^{104-313}$ requires the mitochondrial membrane potential.** Reticulocyte lysate containing ^{35}S-labeled $C^{104-313}$ and isolated *Neurospora* mitochondria were incubated in the presence of ascorbate (*Asc*) plus TMPD (Reaction 1), valinomycin (Reaction 2), and antimycin A (*Anti A*) plus oligomycin (*Oli*) (Reactions 2 and 3) for 25 min at 25 °C as described (11). Reactions were treated with proteinase K (250 μg/ml final concentration). The reisolated mitochondria were analyzed by SDS-polyacrylamide gel electrophoresis. A fluorograph of the dried gel is shown. *C*, imported $C^{104-313}$.

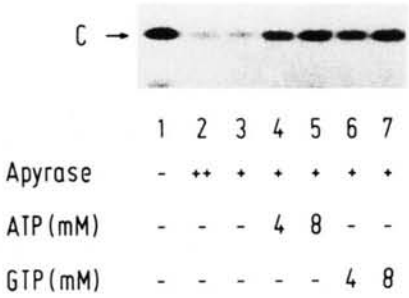

FIG. 2. **Import of $C^{104-313}$ requires NTPs.** Reticulocyte lysate and isolated mitochondria were pretreated with apyrase (30 units/ml in reaction 2 (++); 7 units/ml in Reactions 3–7 (+)) or received an apyrase preparation that had been heated to 95 °C prior to use (corresponding to 30 units/ml; Reaction 1 (−)) as described (18). Reticulocyte lysate and mitochondria were mixed and incubated in the presence of antimycin A, oligomycin, ascorbate, and TMPD. Reactions 4–7 received ATP or GTP as indicated. Import and further treatments were performed as described for Reaction 1 of Fig. 1. *C*, imported $C^{104-313}$.

inhibited (Fig. 1, Reactions 2 and 3). On lysis of mitochondria with Triton X-100, $C^{104-313}$ was completely digested by proteinase K; the precursor in reticulocyte lysate was similarly completely digested by proteinase K (not shown). The import efficiency (*i.e.* ratio between imported precursor and total added precursor) after incubation for 25 min at 25 °C was up to 30% of the import efficiency of the authentic ADP/ATP carrier (10).

Reticulocyte lysate and mitochondria were depleted of ATP and ADP by preincubation with apyrase (an adenosine 5′-triphosphatase and an adenosine 5′-diphosphatase). Reticulocyte lysate and mitochondria were mixed and incubated in the presence of oligomycin and ascorbate plus TMPD as described previously (12, 18). The control reaction that received an apyrase preparation boiled prior to use showed import of $C^{104-313}$ into the protease-protected location (Fig. 2, Reaction 1). Pretreatment with apyrase inhibited the import (Fig. 2, Reactions 2 and 3). Readdition of ATP or GTP restored import (Fig. 2, Reactions 4–7). The import of $C^{104-313}$ therefore requires both $\Delta\psi$ and NTPs.

Specific Binding of $C^{104-313}$ to Mitochondria Occurs in Two Distinct Steps and Requires NTPs—$C^{104-313}$ was bound to mitochondria at 25 °C in the absence of $\Delta\psi$ (addition of antimycin A and oligomycin); the mitochondria were then reisolated and resuspended in BSA-containing buffer (9) in the presence of antimycin A and oligomycin. The reaction mixture was halved. One half received ascorbate plus TMPD to generate a membrane potential. After incubation at 25 °C, both halves were treated with proteinase K (250 μg/ml) to test for imported $C^{104-313}$. The amount of $C^{104-313}$ imported from the bound state (Fig. 3, Reaction 2a) was comparable to the control reaction where ascorbate plus TMPD was present in the first 25 °C incubation (Fig. 3, Reaction 1a). Bound $C^{104-313}$ appeared to be tightly associated with mitochondria since washing of mitochondria prior to import failed to remove the bound precursor (Fig. 3, Reactions 3a and 4a).

The import pathway of authentic ADP/ATP carrier can be

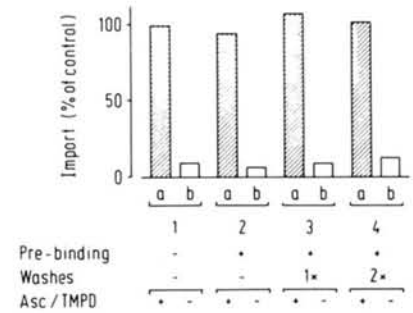

FIG. 3. **$C^{104-313}$ can be imported from the bound state.** Reticulocyte lysate and isolated mitochondria were incubated in the presence of antimycin A and oligomycin for 25 min at 25 °C; Reaction 1a (control) additionally contained ascorbate (*Asc*) plus TMPD. Mitochondria were reisolated and resuspended in BSA-containing buffer in the presence of antimycin A and oligomycin (mix A). Reactions 1 and 2 were left on ice. Mitochondria of Reactions 3 and 4 were washed (once or twice, respectively) in mix A and finally resuspended in mix A. Reactions 2a, 3a, and 4a received ascorbate plus TMPD, and all reactions were incubated for 15 min at 25 °C. After treatment with proteinase K (250 μg/ml final concentration), mitochondria were reisolated and further treated as described in the legend to Fig. 1. Imported $C^{104-313}$ was quantified by densitometry.

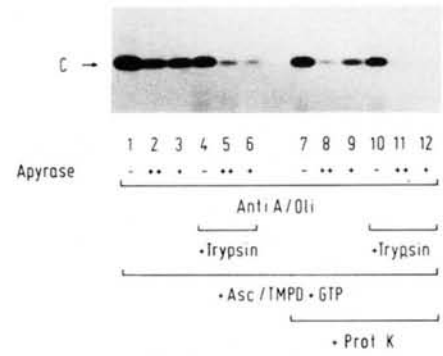

FIG. 4. **Binding of $C^{104-313}$ to mitochondria requires NTPs.** Reticulocyte lysate and mitochondria were incubated with a boiled apyrase preparation (corresponding to 80 units/ml (−)) or active apyrase (80 units/ml (++) or 5 units/ml (+)). Reticulocyte lysate and mitochondria were mixed and incubated in the presence of antimycin A (*Anti A*) and oligomycin (*Oli*) for 25 min at 25 °C. Mitochondria were reisolated and resuspended in buffer containing BSA, antimycin A, oligomycin, and a boiled apyrase preparation (corresponding to 0.25 units/ml (−)) or active apyrase (0.25 units/ml (++ and +)). Reactions were halved, and one half was treated with trypsin (20 μg/ml final concentration). All reactions received ascorbate (*Asc*) plus TMPD and 8 mM GTP and were incubated for 15 min at 25 °C. Reactions were again halved, and one half was treated with proteinase K (*Prot K*, 250 μg/ml final concentration). Mitochondria were reisolated and further treated as described in the legend to Fig. 1. *C*, mitochondria-associated $C^{104-313}$.

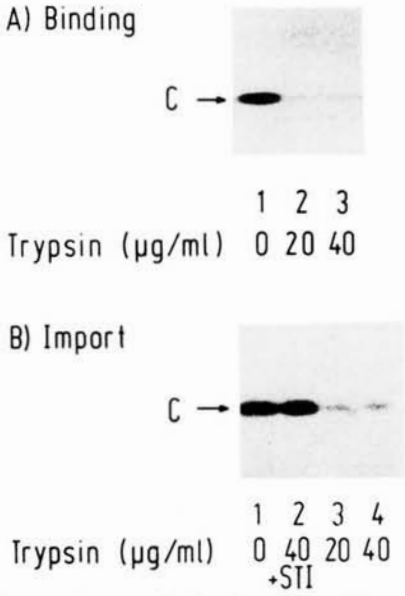

FIG. 5. **Pretreatment of mitochondria with trypsin inhibits binding and import of $C^{104-313}$.** Mitochondria of Reactions 2 and 3 in *panel A* and Reactions 3 and 4 in *panel B* were pretreated with trypsin as described (11); Reaction 2 in *panel B* received trypsin plus soybean trypsin inhibitor (*STI*), and Reactions 1 in *panels A* and *B* received water. Mitochondria were incubated with reticulocyte lysate in the presence of valinomycin, antimycin A, and oligomycin (*panel A*) or in the presence of ascorbate plus TMPD (*panel B*) for 20 min at 25 °C. Reactions were treated with proteinase K (10 μg/ml for *panel A*; 250 μg/ml for *panel B*) and further treated as described in the legend to Fig. 1. *C*, bound or imported $C^{104-313}$.

resolved into several distinct steps (11, 12). Binding of precursor to the mitochondrial surface (generation of the stage 2 intermediate) requires NTPs. Higher levels of NTPs are necessary for insertion of the precursor into the outer membrane (stage 3 intermediate) where it is protected against treatment with trypsin or low concentrations of proteinase K which digest the initial binding sites. Translocation of precursor from the outer membrane into the inner membrane requires $\Delta\psi$, but not NTPs. We followed the import of $C^{104-313}$ to see if it involved the same specific steps. Reticulocyte lysate and mitochondria were either preincubated with a boiled apyrase preparation or received an active apyrase preparation (80 units/ml or 5 units/ml). Reticulocyte lysate and mitochondria were combined and incubated in the absence of $\Delta\psi$ at 25 °C. After reisolation, the mitochondria were resuspended in BSA-containing buffer in the presence of antimycin A, oligomycin, and apyrase. The reaction mixtures were halved, and one half was treated with trypsin to degrade the stage 2 intermediate. NTPs were added and a $\Delta\psi$ was generated by addition of ascorbate plus TMPD. Following incubation at 25 °C, reactions were again halved, and one half was treated with proteinase K (250 μg/ml). Reaction 7 of Fig. 4 shows $C^{104-313}$ which could be imported from the bound state under control conditions. Reaction 10 shows the amount of specifically bound precursor which was protected against treatment with trypsin. Pretreatment of reticulocyte lysate and mitochondria with apyrase reduced the amount of specifically bound precursor (Fig. 4, Reactions 8 and 9) and completely inhibited the generation of the trypsin-protected stage 3 intermediate (Fig. 4, Reactions 11 and 12).

Transport of $C^{104-313}$ from the outer membrane (stage 3 intermediate) to the inner membrane required $\Delta\psi$, but not NTPs; similar to the import of authentic ADP/ATP carrier (12), transport from the outer to the inner membrane was stimulated by removal of NTPs (not shown).

In summary, specific binding of $C^{104-313}$ to the mitochondrial surface and insertion into the outer membrane require NTPs. Translocation of $C^{104-313}$ from the outer into the inner membrane does not need NTPs. Thus, $C^{104-313}$ seems to follow the same specific steps as does the authentic ADP/ATP carrier.

Specific Binding and Import of $C^{104-313}$ Require a Protease-sensitive Component on the Mitochondrial Surface—Mitochondria were pretreated with trypsin at concentrations which do not degrade the outer membrane barrier (8, 11, 20). The mitochondria were then incubated with reticulocyte lysate containing $C^{104-313}$ at 25 °C in the absence (Fig. 5*A*) or presence (Fig. 5*B*) of $\Delta\psi$. Controls received mitochondria which had not been preincubated with trypsin (Fig. 5*A*, Reaction 1; Fig. 5*B*, Reaction 1) or which had been preincubated with trypsin plus trypsin inhibitor (Fig. 5*B*, Reaction 2). Reactions A were treated with proteinase K at a concentration of 10 μg/ml which preserved the stage 3 intermediate. Reactions B were treated with proteinase K at a concentration of 250 μg/ml to test for imported $C^{104-313}$. Pretreatment with trypsin inhibited the specific binding of $C^{104-313}$ (Fig. 5*A*, Reactions 2 and 3) and the import of $C^{104-313}$ (Fig. 5*B*, Reactions 3 and 4). The precursor proteins in reticulocyte lysate were not degraded by incubation with trypsin-pretreated mitochondria (not shown). A protease-sensitive component on the mitochondrial surface, possibly a receptor site, is required at an early stage of import of $C^{104-313}$ into mitochondria.

DISCUSSION

We have shown that the carboxyl-terminal two-thirds (amino acid residues 104–313) of the ADP/ATP carrier polypeptide is imported into mitochondria. There are several interesting reports that artificial (21), nonmitochondrial (22), or cryptic (23) signals or signals contained in a mitochondrial gene product (24) are able to direct proteins into mitochondria with low efficiency. Such import could for example occur as a result of bypassing specific components of the import machinery (*e.g.* receptor sites). To ascertain if the carboxyl-terminal two-thirds of the ADP/ATP carrier contains *specific* mitochondrial targeting information, we investigated the specific steps of the import pathway which are known for the authentic ADP/ATP carrier (7–9, 11, 12, 25). Import of the truncated precursor was mediated by a protease-sensitive component on the mitochondrial surface. Its binding to the outer membrane occurred in two sequential steps; both of them required NTPs. The translocation from the outer membrane into the inner membrane required $\Delta\psi$, but not NTPs. The efficiency of import via the bound state (*i.e.* the ratio between import of precursor via the bound state and import of free precursor) was close to 1 for the truncated precursor and for the authentic ADP/ATP carrier. For all other mitochondrial precursor proteins studied so far, the import efficiency via the bound state was significantly lower (2, 9). This strongly suggests that the truncated precursor contains mitochondrial targeting information specific for the ADP/ATP carrier.

Adrian *et al.* (6) reported that the amino-terminal 115 amino acid residues of the ADP/ATP carrier contained sufficient information for the targeting to mitochondria. So far, we cannot exclude the possibility that the ADP/ATP carrier contains its targeting signal(s) in the region between amino acid residues 104 and 115. However, by studying the published sequence of the ADP/ATP carrier (6, 13, 26), we proposed that it contained multiple internal signal sequences (2); it is composed of three homologous domains of about 100 amino acid residues each (13, 27). The carboxyl-terminal half of each domain contains a positively charged stretch of about 20 amino acid residues which are predicted to form an α-helical

structure (28). Thus, these stretches share characteristics with mitochondrial presequences (29). Dividing the ADP/ATP carrier into three nonoverlapping parts corresponding to the three domains allows testing of the hypothesis that the ADP/ATP carrier contains multiple internal targeting sequences.

The overall efficiency of import (*i.e.* the ratio between imported precursor and total added precursor) of $C^{104-313}$ is up to 30% of the import efficiency of authentic ADP/ATP carrier. This decreased import efficiency may be caused by lack of putative signals present in the amino-terminal third of the ADP/ATP carrier or by an altered conformation of $C^{104-313}$. Further studies have to address the questions of which and how many of the proposed targeting signals are necessary for correct and efficient import of the authentic ADP/ATP carrier.

Our studies show for the first time that an amino-terminal sequence of more than 100 residues is dispensable for specific import of a mitochondrial precursor protein. The general view that specific mitochondrial targeting information is largely confined to the (extreme) amino-terminal region of a mitochondrial precursor protein will have to be reconsidered. These findings for the ADP/ATP carrier are corroborated by studies with a structural mutant of apocytochrome *c* which show that alteration of the carboxyl-terminal precursor part inhibits import of apocytochrome *c* (16). Fusion products of mitochondrial presequences and passenger proteins are often imported with very low efficiency. Taking this into account we should consider the possibility that mature parts of cleavable mitochondrial precursors contain important specific targeting information (besides the obvious role of mature parts for an import-competent conformation (12, 30, 31)). Preliminary results already suggest that the mature part of F_0-ATPase subunit 9 is (directly or indirectly) involved in the $\Delta\psi$-independent specific binding of the subunit 9 precursor to the mitochondrial surface.[2] Thus, the importance of non-amino-terminal precursor regions for specific import into mitochondria may not only be restricted to precursors without cleavable presequences (like the ADP/ATP carrier and apocytochrome *c*).

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[2] N. Pfanner, H. K. Müller, M. A. Harmey, and W. Neupert, unpublished data.

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