Journal of Cellular Biochemistry 36:323–327 (1988)
Molecular Biology of Intracellular Protein
Sorting and Organelle Assembly 93–97

ADP-ATP Carrier of *Saccharomyces cerevisiae* Contains a Mitochondrial Import Signal Between Amino Acids 72 and 111

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The ADP-ATP carrier (also referred to as the adenine nucleotide translocator) of *Saccharomyces cerevisiae* is encoded by a nuclear gene, translated in the cytosol, and imported into the mitochondrial inner membrane. In order to study the determinants of mitochondrial import, a series of fusion proteins, consisting of the first 21, 72, and 111 amino acids of the ADP-ATP carrier, joined to mouse dihydrofolate reductase were generated. Dihydrofate reductase is a cytosolic protein that does not bind mitochondria. The reticulocyte lysate reaction containing the ^{35}S-methionine-labeled protein was incubated with mitochondria in a buffer containing 3% BSA. Following incubation for import, the reactions were treated with 1 mM PMSF or 25 μg/ml proteinase K; mitochondria were reisolated and analyzed by gel electrophoresis. The 21 and 72 amino acid hybrid proteins showed a low level of binding to mitochondria: the bound form was entirely protease accessible. The 111 amino acid hybrid protein was imported to a protease-protected location within mitochondria. It is concluded that the first 72 amino acids of the ADP-ATP carrier do not suffice to import the protein into mitochondria and that the region between amino acids 72 and 111, a region that contains a transmembrane-spanning domain, constitutes at least part of the mitochondrial import signal.

Key words: intracellular sorting signal, mitochondrial inner membrane protein, in vitro import

Localization of newly translated proteins to intracellular organelles requires a signal in the primary sequence of the protein and a mechanism for recognizing the signal in the organelle. Mitochondrial import signals previously identified have been generally characterized by the presence of a basic N-terminal region, which by itself suffices to direct proteins to the mitochondrial matrix. The presence of a hydrophobic sequence and the distance of the sequence from the matrix-import sequence appear to determine to which of the intramitochondrial compartments the protein localizes [1–3].

Received May 12, 1987; revised and accepted October 13, 1987.

The *Saccharomyces cerevisiae* ADP-ATP carrier (AAC) is a 309 amino acid protein [5] encoded by a nuclear gene isolated by the complementation of the *PET9* mutation [4]. The AAC protein, which is predicted to contain four transmembrane-spanning domains, lacks a cleaved presequence and contains approximately equal numbers of basic and acidic residues in the N-terminal region. The first 115 amino acids of the ADP-ATP carrier suffice to import the β-galactosidase fusion protein into a protease-protected location in transformed intact yeast cells [5].

The present in vitro import results indicate that the first 72 amino acids of the ADP-ATP carrier do not constitute a mitochondrial import signal when fused to the cytosolic protein, mouse dihydrofolate reductase (DHFR). The inclusion of the region between amino acids 72 and 111, a region that contains a hydrophobic transmembrane-spanning domain (amino acids 72–98), does result in import of the DHFR fusion protein to a protease-protected location in vitro.

METHODS

Plasmids

The yeast *PET9* gene, derived from pBR6-19-28 [5], and mouse DHFR DNA, derived from pFD11 [6], were inserted into a pT7 transcription vector (Genescribe).

Generation of the ADP-ATP Carrier-DHFR Fusion Proteins

The Hind III-Sal I fragment of pFD11 [6] encoding full-length DHFR was moved into a pT7 vector (Genescribe) containing the promoter for T7 RNA polymerase. The Eco RI-Sst I fragment of TZ115 [5] containing 345 bp of the 5′ coding region of the ADP-ATP carrier and 2 kb of *lac z* was moved into pUC19 (International Biotechnologies, Inc.). The Hind III fragment of the resulting construct was moved in the Hind III site upstream of DHFR in the pT7-DHFR vector, generating a construct that was subsequently linearized at the unique Bam HI site and digested with Bal 31 endonuclease. The remaining *lac z* DNA was released by using the Pst I site derived from pUC19. Following treatment with T4 DNA polymerase the vector was ligated with T4 ligase to form the pTD vector series (Smagula and Douglas, in press), in which variable lengths of the ADP-ATP carrier are fused to DHFR (Fig. 1). Transcription by T7 polymerase was followed by translation in reticulocyte lysate (Promega Biotec) in the presence of ^{35}S-methionine [7]. pTD constructs that translated as proteins larger than DHFR as shown by SDS–acrylamide gel analysis [8] were sequenced by the dideoxy method [9]. Translation reactions to be used for in vitro import studies were frozen in liquid N^2 and stored at −70°C. Techniques used were as described by manufacturers or by Maniatis et al. [10].

Yeast Strain, Media

Mitochondria were isolated from wild-type yeast strain D273-10B (MATα), grown in semisynthetic salts 2% lactate medium [11] to O.D. (600 nm) 1.2.

In Vitro Import

Isolated mitochondria were suspended in 250 mM sucrose, 1 mM EDTA, 10 mM MOPS/KOH pH 7.2 [12] to a concentration of 10–20 μg protein/μl. Reticulocyte lysate containing the ^{35}S-methionine-labeled protein was incubated with 100–200 μg mitochondria in a buffer containing 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 10

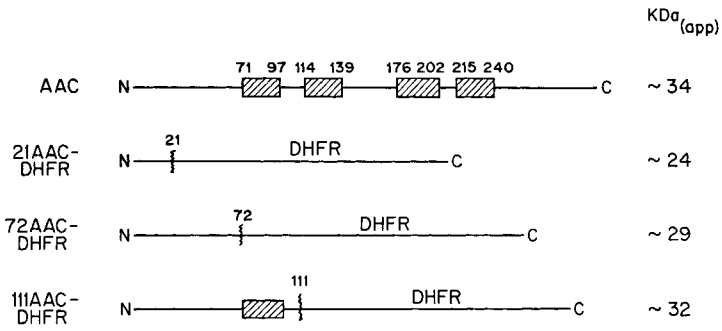

Fig. 1. AAC-DHFR constructs. The location of transmembrane-spanning hydrophobic domains is indicated by shaded regions. Approximate molecular weight of proteins is indicated at right. The location of the fusion junction is shown for each construct.

mM MOPS/KOH pH 7.2, and 3% BSA [12] in a total volume of 200 μl. Import reactions were incubated at 25°C for 25 min, cooled on ice for 5 min, divided in half, and had 1 mM PMSF or 25 μg/ml proteinase K added to them. After 30 min at 0°C, 1 mM PMSF was added to the proteinase-K-treated reaction and incubation at 0°C continued for 5 min. Mitochondria were reisolated through 0.6 ml 20% sucrose 10 mM Tris-Cl pH 7.4, and the pellet was analyzed by gel electrophoresis [8]. The gel was exposed to Kodak X-AR film at −70°C.

RESULTS

Fusion proteins 21-DHFR, 72-DHFR, and 111-DHFR, consisting of 21, 72, and 111 amino acids of the ADP-ATP carrier upstream of DHFR, were selected for in vitro import studies. The hybrid proteins encoded by the pTD vector series (Fig. 1) consist of various lengths of the carrier separated from DHFR by the 12 amino acid sequence XHASLSILEFAI (Fig. 1).

To a slight degree 21-DHFR and 72-DHFR bind mitochondria (Fig. 2, 21-DHFR, 72-DHFR, lane 2). When mitochondria with bound 21-DHFR or 72-DHFR are treated with 25 μg/ml proteinase k, both hybrid proteins are completely digested, indicating that they are bound to the mitochondrial surface (lane 3). The 111-DHFR protein both binds mitochondria and is imported to a protease-protected location following treatment of the import reaction with 25 μg/ml proteinase K (Fig. 2, 111-DHFR, lanes 2 and 3). Two smaller proteins, probably translated from internal methionines in the first 111 amino acids of the ADP-ATP carrier (lane 1, the two bands immediately below 111-DHFR), also bind mitochondria and are imported. The DHFR that is present in the 111-DHFR reticulocyte lysate (lane 1, lower band) due to translation from the DHFR start codon that is retained in the construct does not coisolate with mitochondria. DHFR alone does not bind to mitochondria under these conditions (Fig. 2, DHFR, lane 2).

DISCUSSION

Previous work has shown that the first 115 amino acids of the ADP-ATP carrier fused to β-galactosidase suffice to import the fusion protein to a protease-protected

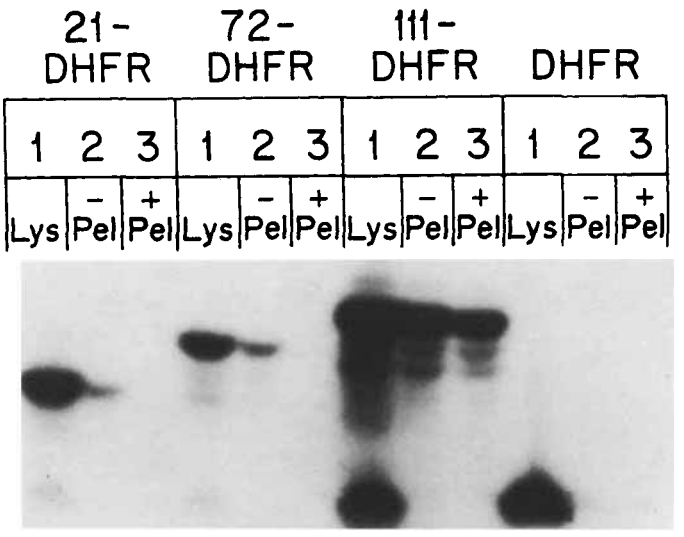

Fig. 2. Binding and import of 21-DHFR, 72-DHFR, 111-DHFR, and DHFR. **Lane 1:** 20% of reticulocyte lysate containing ^{35}S-labeled protein added to import reaction. **Lane 2:** 1 mM PMSF added following import, mitochondria reisolated. **Lane 3:** Import reaction treated with 25 μg/ml proteinase K following import, mitochondria reisolated. Import buffer contained 3% BSA [12].

location in transformed yeast cells [5]. The present results, based on in vitro import of DHFR fusion proteins, indicate the absence of an import signal in the first 72 amino acids of the carrier and the presence of an import signal in the region between amino acids 72 and 111. The absence of an import signal in the extreme N-terminal region suggests that the ADP-ATP carrier is imported by a different mechanism than mitochondrial proteins that contain an import signal in the N-terminal region. Evidence that binding and import of porin and the ADP-ATP carrier of *Neurospora crassa* are blocked by protease treatment of mitochondria under conditions that do not hinder import of ATPase subunits 2 and 9 suggests that import of the carrier and of porin is mediated by a common component that is distinct from components that mediate import of the ATPase subunits [13]. The fact that water-soluble porin competes for the binding site of the ADP-ATP carrier of *Neurospora crassa* [14] reinforces the possibility that the first step of import of the ADP-ATP carrier [12] may be identical with that of porin.

ACKNOWLEDGMENTS

This work is supported by NIH GM 36536 and I-814 from the Robert A. Welch Foundation.

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