Role of the N- and C-termini of porin in import into the outer membrane of *Neurospora* mitochondria

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Received 10 May 1996; revised version received 31 May 1996

Abstract The signals for targeting and assembly of porin, a protein of the mitochondrial outer membrane, have not been clearly defined. Targeting information has been hypothesized to be contained in the N-terminus, which may form an amphipathic α-helix, and in the C-terminal portion of the protein. Here, the role of the extreme N- and C-termini of porin from *Neurospora crassa* in its import into the mitochondrial outer membrane was investigated. Deletion mutants were constructed which lacked the N-terminal 12 or 20 residues or the C-terminal 15 residues. The porins truncated at their N-termini were imported in a receptor-dependent manner into the outer membrane of isolated mitochondria. When integrated into the outer membrane, these preproteins displayed an increased sensitivity to protease as compared to wild-type porin. In contrast, mutant porin truncated at its C-terminus did not acquire protease resistance upon incubation with mitochondria. Thus, unlike most other mitochondrial preproteins, porin appears to contain important targeting and/or assembly information at its C-terminus, rather than at the N-terminus.

Key words: Porin; Mitochondria; Protein import; Outer membrane; Targeting signal; Membrane insertion

1. Introduction

Nuclear-encoded mitochondrial preproteins are synthesized in the cytoplasm and harbor signals which are essential for their subsequent import into the organelle [1–4]. Protein translocation into and across the two mitochondrial membranes is achieved through the concerted action of two protein translocation complexes: the TOM complex (translocase of the outer membrane of mitochondria; reviewed in [5]) and the TIM complex (translocase of the inner membrane of mitochondria; [6]; reviewed in [7]). Targeting and initial translocation of most proteins destined for the matrix are dependent on an amino-terminal, cleavable presequence which may adopt an amphipathic, α-helical structure [8–10]. In contrast, all proteins of the outer membrane, and several of those of the inner membrane and the intermembrane space, contain non-cleavable targeting signals. The location of these signals is known for only a few preproteins. In Tom70, a receptor component of the TOM complex, the targeting sequence includes the amino-terminal membrane anchor of the protein [11,12]. In the outer membrane protein Bcl-2, both of these functional segments lie at the C-terminus [13]. Bcs1, an integral inner membrane protein [14], contains an internal signal located on the C-terminal side of the single trans-membrane domain [15]. Thus, the import signals in at least some of the proteins with a single membrane-spanning segment are located in the vicinity of the membrane anchor.

The mitochondrial targeting signals in multi-topic membrane proteins, such as porin, are unknown [16]. Mitochondrial porins are predicted to traverse the outer membrane as a series of anti-parallel β-strands (reviewed in [17]). The amino-terminal residues of porin have the potential to form an amphipathic α-helix [17,18], and may lie at one surface of the membrane [19]. This region of the protein is not essential for either pore formation or voltage-dependent gating [20]. However, a potential role for this sequence as an import signal was suggested on the basis of its similarity to mitochondrial presequences and to the amino-terminus of Tom70 [18,21]. The N-termini of porins differ from cleavable presequences in that they possess both positively and negatively charged residues (Fig. 1). Deletion of residues 17–98 [22] or 9–156 [23] of yeast porin abrogates import. However, it could not be excluded that structural changes prevented assembly of the mutant proteins into a protease-resistant state in the membrane.

Other studies have implicated a role for the C-terminal region of porin in targeting and assembly. The import efficiency of the 283-residue yeast porin is decreased when one of two residues, Lys234 and Lys236, is mutated to a neutral or negatively charged amino acid [23]. Additionally, deletion of the last 62 residues of yeast porin prevents its import [22]. Clearly, more precise deletions at both the N- and C-termini are required to define the roles of these regions in the import process. In the present study we tested the requirements for the N-terminal 20 and the C-terminal 15 residues of *Neurospora* porin in its import into the outer membrane of mitochondria.

2. Materials and methods

2.1. Construction of porin mutants

The porin mutants used in this study were constructed as described previously [20] and cloned into the vector pGEM4 (Promega). Briefly, restriction fragments of the *Neurospora* porin cDNA clone [18] were replaced by PCR products lacking either amino acid residues 2–12 or 3–20, to create Δ2–12porin and Δ3–20porin, respectively (Fig. 1). Mutations in the PCR-generated fragments were excluded by DNA sequencing. Δ269–283porin was created by deleting a *Hin*dIII fragment of the original cDNA clone. This resulted in the removal of amino acids 269–283 and the addition of a single glutamine residue at the C-terminus (Fig. 1). The stop codon was encoded by the vector.

2.2. Import of porin into Neurospora mitochondria

The standard laboratory strain, 74A, of *Neurospora crassa* was

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PII S0014-5793(96)00629-1

grown and maintained under standard conditions [24]. Mitochondria were freshly isolated prior to each import experiment [25]. Radiolabelled porin preproteins were synthesized by coupled in vitro transcription/translation (TNT SP6 coupled reticulocyte lysate system, Promega) in the presence of [^{35}S]methionine (ICN Radiochemicals) as label. These preproteins (2–10 μl) were imported into the isolated mitochondria (30 μg) for 7 min at 25°C in 100 μl of import buffer (250 mM sucrose, 30 mg/ml bovine serum albumin, 5 mM $MgCl_2$, 80 mM KCl, 2 mM ATP, 2 mM NADH, 10 mM MOPS, pH 7.2 [26]). Following import, the samples were chilled on ice and, where indicated, were treated with various concentrations of proteinase K for 15 min on ice. The protease digestion was stopped by the addition of phenylmethylsulfonylfluoride (0.5 mM final concentration). Alternatively, the reisolated mitochondria were washed with alkaline buffer (100 mM Na_2CO_3, pH 11.5), and the membranes were collected by centrifugation at 100 000 × *g* for 1 h at 2°C. In some experiments, mitochondria in SEM buffer (220 mM sucrose, 1 mM EDTA, 10 mM MOPS, pH 7.2) were pretreated with trypsin (20 μg/ml [27]) to remove surface-exposed regions of the outer membrane import machinery. To specifically block the surface receptors, mitochondria in import buffer (lacking ATP and NADH) were preincubated for 40 min with immunoglobulins G (IgGs; 5 μg/μg mitochondrial protein [28]) directed against Tom20, or derived from preimmune serum. Following dilution in SEM buffer, the organelles were reisolated by centrifugation and used in standard import reactions. In all cases, the resulting samples were analyzed by SDS-PAGE and autoradiography.

2.3. *Miscellaneous procedures*

Published or manufacturer's protocols were used for the following: standard DNA manipulations [29]; raising of antisera and purification of IgGs [28]; blotting of proteins onto nitrocellulose and immunostaining with the ECL chemiluminescence detection system (Amersham), and quantitation of bands on X-ray films with either a Pharmacia Image Master densitometer or a Fujifilm BAS-1500 Bioimaging analyzer.

3. Results

To investigate the role of the N-terminus of porin in its import, deletion mutants of *Neurospora* mitochondrial porin were generated which lacked either residues 2–12 (Δ2–12porin) or residues 3–20 (Δ3–20porin) (Fig. 1). Radioactively labelled preproteins were synthesized by in vitro coupled transcription/translation and imported into isolated *Neurospora* mitochondria. Import was assayed by treating the samples with various concentrations of proteinase K after the reaction. Upon import and assembly into the outer membrane, wild-type porin becomes completely resistant to high levels of protease (100 μg/ml [30]; Fig. 2A). With the mutant porins, full-length molecules, as well as several smaller protease-protected fragments, were detected. These protease-resistant species were not generated from either Δ2–12porin or Δ3–20porin upon incubation with trypsin-pretreated mitochondria (Fig. 2A), or in the absence of mitochondria (not shown). Thus, the fragments resulted from preproteins that were imported into the organelle in a receptor-dependent manner. The N-terminally truncated preproteins were more sensitive to proteinase K than is the wild-type porin (Fig. 2A), and, at higher concentrations of protease, gave rise to a series of fragments, the smallest of which had an apparent molecular mass of about 22 kDa. The digestion patterns of the two mutants were alike, suggesting that cleavage occurred at the N-terminus to produce C-terminal fragments of identical electrophoretic mobility.

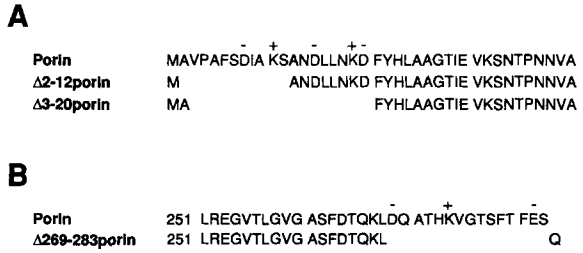


Fig. 1. Truncated porin molecules. (A) N-terminal truncations. The sequences of the amino-terminal regions of wild-type porin, and the deletion mutants Δ2–12porin and Δ3–20porin are given. (B) C-terminal truncation. The C-terminal regions of wild-type porin and Δ269–283porin are presented. The C-terminal glutamine residue (Q) in the latter sequence is encoded by the vector.

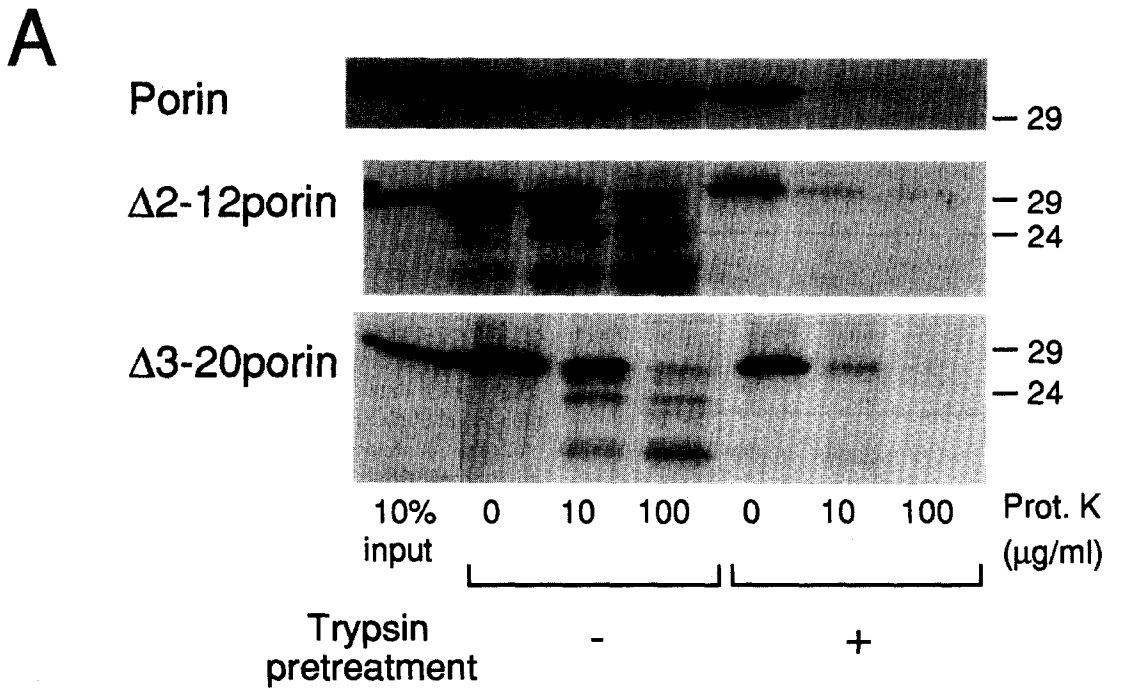
To ascertain whether Δ2–12porin and Δ3–20porin, like the wild-type protein, utilize the TOM complex during their import, these preproteins were imported into mitochondria that were pretreated with IgGs raised against Tom20, a receptor component that is utilized by porin [25,28]. The import of the N-terminally truncated porins was inhibited by these IgGs (Fig. 2B), establishing that porin lacking the N-terminus follows the normal import route into the outer membrane.

The insertion of the mutant porins into the lipid bilayer of the outer membrane can be tested by following their resistance to alkaline extraction after the import reaction. This process removes proteins which are not integrated into the membrane [31,32] (Fig. 3). Mitochondria containing freshly imported porins were washed with alkaline buffer, the membranes were collected by centrifugation and the proteins in both the pellet and supernatant fractions were analyzed. A fraction of wild-type porin and of both of the N-terminally-truncated proteins remained associated with the membranes after this treatment, indicating that they were integrated into the lipid bilayer (Fig. 3). By this criterion, the membrane insertion of the mutant porins is about half as efficient as that of the wild-type protein.

We also tested the import competence of mutant porin truncated at its C-terminus. Δ269–283porin forms stable, voltage-gated pores upon insertion into artificial bilayers [20], suggesting that it could become imported into mitochondria along the physiological pathway and form channels. However, in contrast to the N-terminal deletion porins described above, only a minor fraction of Δ269–283porin acquired resistance to low levels of protease upon incubation with intact mitochondria (Fig. 4). Most of this protease-resistant material was also observed after incubation with trypsin-pretreated organelles. The Δ269–283porin that associated with mitochondria in the absence of protease digestion was almost completely susceptible to alkaline extraction (Fig. 3). Together, these data indicate that hardly any Δ269–283porin became integrated into the outer membrane. Thus, the C-terminus of porin is required for the import and/or proper assembly of the protein into the outer membrane.

4. Discussion

Our studies demonstrate that the N-terminus of porin is not an essential mitochondrial targeting signal. Both Δ2–12porin and Δ3–20porin are imported into the outer membrane as indicated by both protease digestion and alkaline extraction experiments. The import of these preproteins is prevented by trypsin-pretreatment of the mitochondria and by blocking Tom20 with IgGs. This suggests that, despite its similarity to mitochondrial presequences, the N-terminus of the porin precursor does not interact with the Tom20-Tom22 receptor



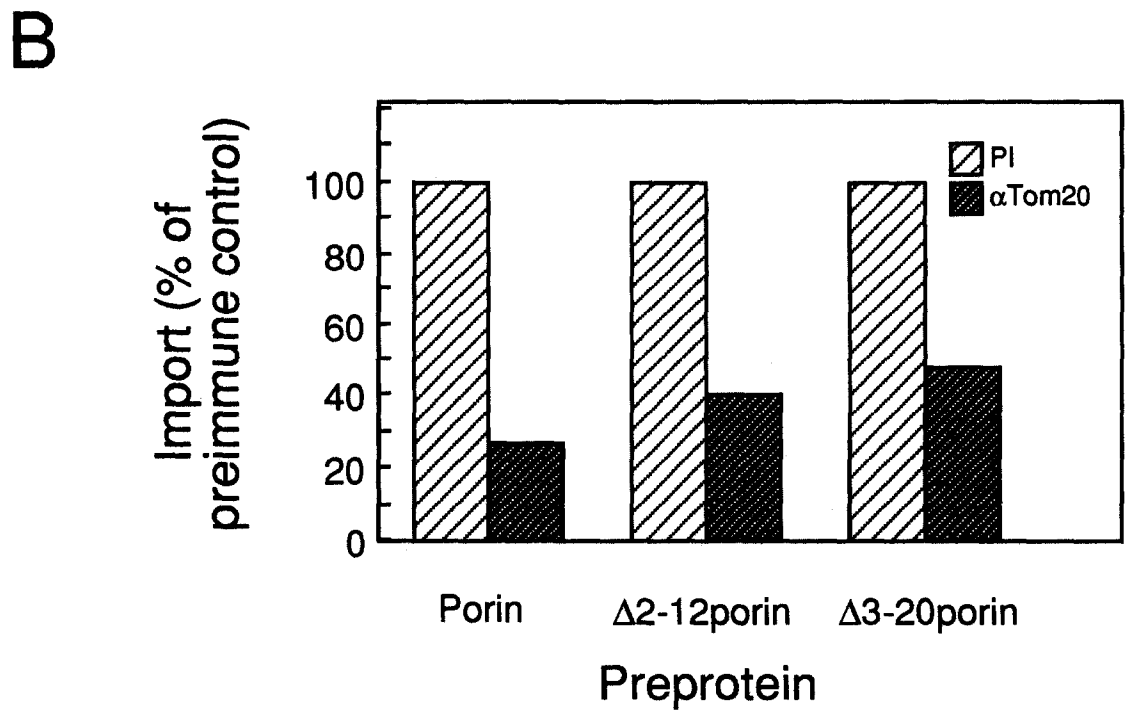


Fig. 2. Import of truncated porins into isolated *Neurospora* mitochondria. (A) Wild-type porin, Δ2–12porin and Δ3–20porin were synthesized by in vitro transcription/translation in the presence of [^{35}S]methionine. The proteins were imported into isolated organelles. The mitochondria were pretreated with trypsin in the presence or absence of soybean trypsin inhibitor (20-fold w/w excess) prior to their inclusion in the reaction mixture. Following import, the samples were chilled on ice and proteinase K was added to the indicated final concentrations. The organelles were reisolated by centrifugation and subjected to SDS-PAGE, followed by autoradiography of the dried gel. The positions of the molecular mass markers are indicated on the right of the figure. A standard representing 10% of the radiolabelled preprotein added to the import reaction was included on the gel (10% input). (B) Inhibition of porin import by IgGs against Tom20. Mitochondria were preincubated with IgGs derived from preimmune serum (PI) or raised against Tom20 (αTom20). The organelles were used for standard import reactions as described above and the samples were digested with 10 μg/ml proteinase K prior to analysis by SDS-PAGE. The radioactive signals were quantitated and, for each preprotein, the level of import in the presence of IgGs derived from preimmune serum was set at 100%. The experiments were performed three times and the average data are presented. The standard error was 15%. The presence of IgGs derived from pre-immune serum does not reduce the import of porin by more than 10% (data not shown; cf. [34]).

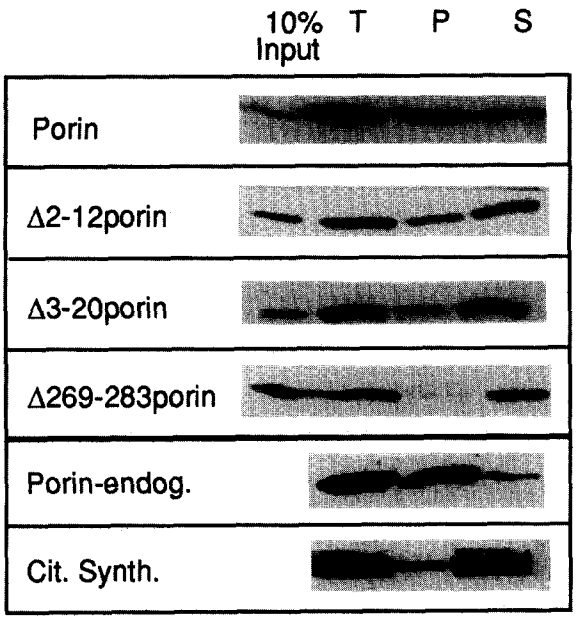


Fig. 3. Membrane insertion of imported porin derivatives. Standard import reactions were carried out as described for Fig. 2. Following import, the samples were chilled on ice, and diluted 10-fold with SEM buffer. The samples were split into two parts and the mitochondria were reisolated by centrifugation. One of the mitochondrial pellets was resuspended in SDS-PAGE sample buffer and represents the total (T) protein associated with the organelles. Mitochondria in the second portion were resuspended in carbonate buffer (0.1 M Na_2CO_3, pH 11.5) and incubated on ice for 30 min. Membranes were then collected by centrifugation and the proteins in the supernatant (S) were precipitated with trichloroacetic acid and were analyzed by SDS-PAGE alongside the membrane pellet (P) and the total untreated mitochondria (T). The gel was blotted to nitrocellulose and exposed to an X-ray film. The procedure was controlled by immunostaining the blot for endogenous porin (Porin-endog.), and a soluble matrix protein, citrate synthase (Cit. Synth.).

unit. These components of the TOM complex have been shown to recognize the N-terminal mitochondrial presequences of matrix-targeted preproteins [33]. The absence of the N-terminal segment results in a decreased import efficiency. However, this reduction is only two-fold and thus differs significantly from the total lack of import of matrix-targeted preproteins in the absence of their N-terminal presequences (reviewed in [10]). The decreased import efficiency of the N-terminally truncated porins could result from folding into sub-optimal secondary or tertiary structures after synthesis in reticulocyte lysate, or at a particular stage of preprotein recognition, import or assembly into the membrane.

The truncated porins display an increased protease sensitivity upon import, but remain resistant to carbonate. Therefore, their increased susceptibility to degradation by protease is not due to an inability to insert into the lipid bilayer. Rather, the truncated proteins seem to adopt a conformation in the membrane that renders them more sensitive to proteolytic attack. In support of this hypothesis, we have observed that porin molecules lacking the N-terminus form unstable channels which rapidly fluctuate between numerous open substates [20].

The extreme C-terminus of *Neurospora* porin is required for its import into mitochondria. This result is intriguing, especially given that Δ269–283porin can form voltage-gated channels in artificial bilayers [20]. Thus, the C-terminus of porin contains a sequence that is essential for insertion into the membrane along the physiological pathway. The last 15 residues of *Neurospora* porin have a net charge of −1, unlike the positively charged presequences recognized by the Tom20-Tom22 receptor unit. Therefore, we consider it rather unlikely that the C-terminus of porin represents a novel type of targeting signal. Alternatively, misfolding of Δ269–283porin prior to an interaction with the TOM complex might sequester the targeting information that exists elsewhere in the molecule. The C-terminal sequence of porin is proposed to include one or two trans-membrane β-strands (reviewed in [17]) and its absence may prevent normal assembly of the protein into the membrane following an interaction with, or release from, the import machinery.

For porin, it is now clear that the targeting information is not located at the N-terminus, but in other regions of the protein, which include its extreme C-terminus. However, evidence is accumulating that a single stretch of amino acids does not suffice as a targeting signal, because point mutations in certain charged residues [23], as well as larger internal deletions [22,23], influence the efficiency of import. Thus, in contrast to proteins which span the membrane once, targeting signals in multi-topic mitochondrial proteins, such as porin, are not comprised of short segments of these polypeptides. Several regions localized in different parts of the protein influence the import efficiency, suggesting that structural elements of the preprotein or the folding state during the import reaction may represent (part of) the targeting information. Therefore, it may be difficult to fully separate the targeting and assembly signals in multi-topic proteins.

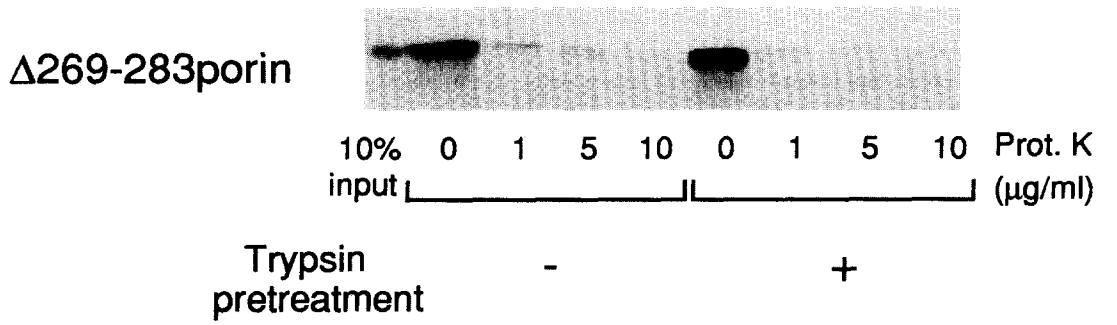


Fig. 4. Import of Δ269–283porin into mitochondria. The preprotein was imported and samples were analyzed as described for Fig. 2. Note that lower concentrations of proteinase K (Prot. K) were used in the subsequent treatment of the mitochondria.

Acknowledgements: The expert technical assistance of M. Braun and P. Heckmeyer is gratefully acknowledged. This research was supported by the Sonderforschungsbereich 184 of the Deutsche Forschungsgemeinschaft, the Fonds der Chemischen Industrie and a post-doctoral fellowship from the Medical Research Council of Canada to D.A.C.

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