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C- to N-terminal translocation of preproteins into mitochondria

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Nuclear-encoded mitochondrial matrix proteins in most cases contain N-terminal targeting signals and are imported in a linear N- to C-terminal (N→C) fashion. We asked whether import can also occur in a C- to N-terminal direction (C→N). We placed targeting signals at the C-terminus of passenger proteins. Import did occur in this 'backwards' fashion. It paralleled that of the 'normal' N→C mechanism in terms of efficiency, rate, energetic requirements and ability to mediate unfolding and refolding during and following import of protein containing a folded domain. Furthermore, this reaction was mediated by the TIM17–23 machinery. The import pathway taken by certain inner-membrane proteins contains elements of such a C→N translocation pathway, as they are targeted to mitochondria by internal targeting signals. These internal targeting signals appear to form loop structures together with neighbouring transmembrane segments, and penetrate the inner membrane in a membrane-potential-dependent manner. The dimeric TIM17–23 complex, together with mt-Hsp70, acts on both sides of the loop structure to facilitate their translocation into the matrix. On one side of the loop import occurs in the common N→C direction, whereas the translocation of the other side involves the novel C→N import direction. We conclude therefore that the mitochondrial import machinery displays no preference for the directionality of the import process.

Keywords: mitochondria/protein import/reverse translocation/yeast

Introduction

The import of nuclear-encoded precursor proteins into mitochondria is mediated by the translocases in the outer membrane (the TOM complex) and the inner membrane (the TIM complexes) (for review see Schatz and Dobberstein, 1996; Neupert, 1997; Pfanner and Meijer, 1997). The TIM17–23 complex (Dekker *et al*., 1993; Emtage and Jensen, 1993; Maarse *et al*., 1994; Berthold *et al*., 1995; Bauer *et al*., 1996) mediates the translocation of presequence-targeted proteins across the inner membrane and acts in cooperation with Tim44 and the ATP-driven molecular chaperone mt-Hsp70 (Maarse *et al*., 1992; Horst *et al*., 1993; Kronidou *et al*., 1994; Rassow *et al*., 1994; Schneider *et al*., 1994, 1996; Ungermann *et al*., 1996). The majority of nuclear-encoded proteins destined for the mitochondria are synthesized as preproteins containing N-terminal cleavable presequences. These positively charged presequences, known also as mitochondrial targeting signals (MTS), display the potential to form amphipathic α-helices (von Heijne, 1986). They serve initially to target the preproteins to the mitochondria, where they are recognized by receptor proteins on the surface. The N-terminal presequences perform a second important function by initiating the $\Delta\psi$-dependent translocation of the polypeptide across the inner membrane into the matrix. Consequently, preproteins with such presequences are considered to be imported across the mitochondrial membranes in a linear and N- to C-terminal (N→C) fashion. Import in this manner has been confirmed by arresting intermediates with an N_{in}–C_{out} topology spanning both the outer and inner membranes (Schleyer and Neupert, 1985). Following import into the matrix, N-terminal presequences are proteolytically removed by the mitochondrial processing peptidase (MPP) (Böhni *et al*., 1983; Schmidt *et al*., 1984).

A recent analysis of import of the inner-membrane protein Bcs1p has indicated that not all nuclear-encoded proteins are imported in such a linear fashion (Fölsch *et al*., 1996). The Bcs1p spans the inner membrane with a single transmembrane segment in an N_{out}–C_{in} orientation. It is synthesized as a preprotein without an N-terminal presequence. A segment of positively charged amino acids located directly C-terminal to the single transmembrane domain has the potential to form an amphipathic helix. Together with the transmembrane domain, it functions as an internal targeting signal, presumably by forming a hairpin loop structure. Translocation of this complex targeting signal into the inner membrane occurs through the TIM17–23 machinery (Fölsch *et al*., 1996; our unpublished results). Completion of import of the Bcs1p is driven by the ATP-dependent action of mt-Hsp70 (Fölsch *et al*., 1996). The N-terminal domain of Bcs1p becomes sorted by an unknown mechanism to the intermembrane space, to result in an N_{out}–C_{in} orientation in the inner membrane. Extension of the N-terminal tail of Bcs1p by the addition of a dihydrofolate reductase (DHFR) moiety (DHFR–Bcs1p) prevented its correct sorting and caused accumulation of the resulting protein in the matrix (Fölsch *et al*., 1996). Thus, longer polypeptide segments present N-terminal to an internal mitochondrial targeting signal are capable of being efficiently imported in a C- to N-terminal (C→N) direction. However, the reason for the complete import into the matrix when the N-terminal tail is extended is not yet clear.

This observation raises the question as to whether hydrophilic proteins can be imported into the matrix if

they carry a classical MTS at their C-terminus. And if so, how would this C→N import pathway be mediated? These questions have a number of important implications on the function of the TOM and TIM complexes. In addition, the existence of a C-terminal directed import pathway opens the possibility that there may be mitochondrial proteins which do not have the classical N-terminal MTS signatures, but rather C-terminal ones.

We demonstrate here that the N-terminal region of a looped internal targeting signal becomes imported across the TIM channel in a mt-Hsp70 dependent fashion in a C→N direction. Positively charged MTS derived either from the Bcs1p or from an authentic mitochondrial N-terminal presequence, placed at the C-terminus of DHFR, can mediate efficient import into mitochondrial matrix in a linear, but backwards C→N, direction. Import in this manner occurs via the TIM17–23 import complex and requires the co-ordinate action of the ATP-driven mt-Hsp70.

Results

Translocation of polypeptide segments N-terminal to an internal mitochondrial targeting signal

We investigated the mechanism of translocation across the inner membrane of segments of a polypeptide segment located N-terminal to an internal targeting signal. A number of proteins were constructed by fusing mouse cytosolic dihydrofolate reductase (DHFR) to N-terminal regions of Bcs1p (Figure 1A). They were then imported into isolated mitochondria. In this analysis, we took advantage of our previous observation that the placement of a DHFR moiety at the N-terminus of Bcs1p results in the missorting of the complete protein into the matrix in a $\Delta\psi$-dependent manner (Figure 1B; Fölsch *et al*., 1996).

In the chimera DHFR–Bcs1p(1–83) (Figure 1A), the bulk of the C-terminal domain of Bcs1p was deleted, leaving the internal targeting signal, consisting of the transmembrane domain and the positively charged segment, exposed at the C-terminus (Figure 1A). Radiolabelled DHFR–Bcs1p(1–83) was efficiently imported into isolated mitochondria, where it was located in the matrix, as revealed by proteinase K (PK) treatment of the samples under hypotonic swelling conditions (Figure 1B). Import was strictly dependent on the presence of a membrane potential. A substantial portion of the imported DHFR–Bcs1p(1–83) was processed to a slightly smaller species. This processing is probably catalysed by the mitochondrial processing peptidase (MPP); it was sensitive to the presence of the metal chelators EDTA and *o*-phenanthroline and was not inhibited following import into mitochondria isolated from yeast mutants deficient in one of the other known mitochondrial proteases, Yta10p, Yta12p, Yme1p or Pim1p (results not shown). In fact, processing by MPP at internal sites in a polypeptide, rather than N-terminally, has been reported previously for an authentic matrix protein (Gessert *et al*., 1994). It is therefore conceivable that C-terminal processing of DHFR–Bcs1p(1–83) by MPP may occur. Furthermore, analysis of the size of the DHFR moiety of the imported DHFR–Bcs1p(1–83) indicated that the N-terminus of DHFR had not undergone processing, confirming that the observed cleavage occurred at the C-terminus of the protein (see discussion of Figure 3C).

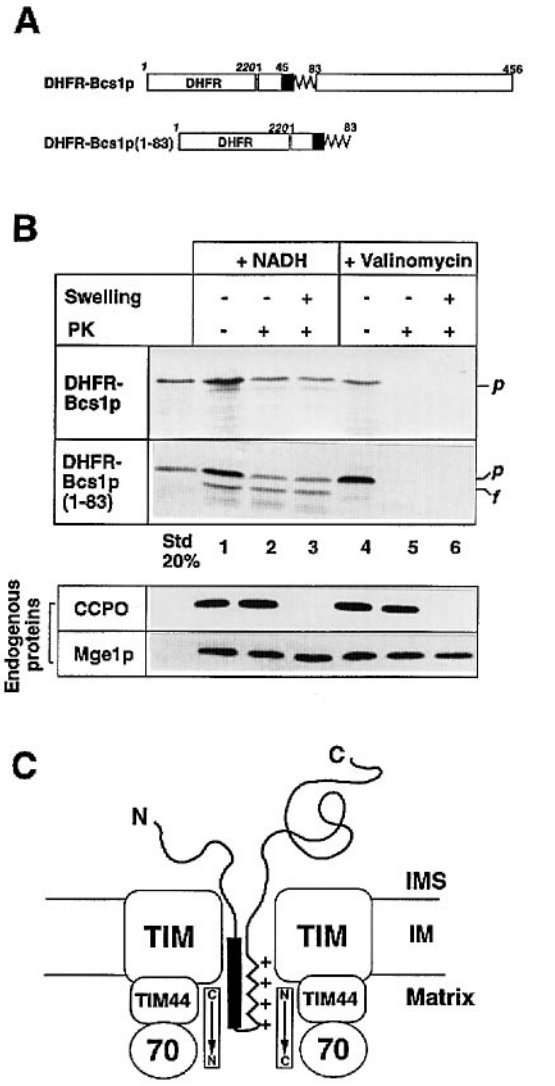

Fig. 1. Import of DHFR–Bcs1p-derived fusion proteins. (**A**) DHFR–Bcs1p fusion proteins. Black areas denote the single transmembrane domain (amino acids 45–68) and zig-zag lines denote the targeting sequence (amino acids 69–83). (**B**) Radiolabelled proteins were imported into isolated mitochondria for 10 min at 25°C, in the presence (+ NADH) or absence (+ Valinomycin) of a membrane potential. Mitochondria were reisolated and were either mock-treated or subjected to PK treatment under non-swelling or swelling conditions, as described in Materials and methods. Samples were analysed by SDS–PAGE and blotted onto nitrocellulose. Immunodecoration of marker proteins cytochrome *c* peroxidase (CCPO) (intermembrane space) and Mge1p (matrix) was performed; Std 20%, 20% of the amount of radiolabelled protein added to each reaction; p, non-processed species; f, MPP-processed species. (**C**) Model depicting the active translocation across the TIM17–23 (TIM) import machinery of both sides of a looped targeting signal, driven by Tim44/mt-Hsp70.

In summary, translocation of polypeptide segments N-terminal to an internal targeting signal of a preprotein does not require the concomitant translocation of larger C-terminal regions. Therefore, N-terminal segments can become imported in an active manner in a C→N fashion (rather than by a passive random diffusion process) (Figure 1C).

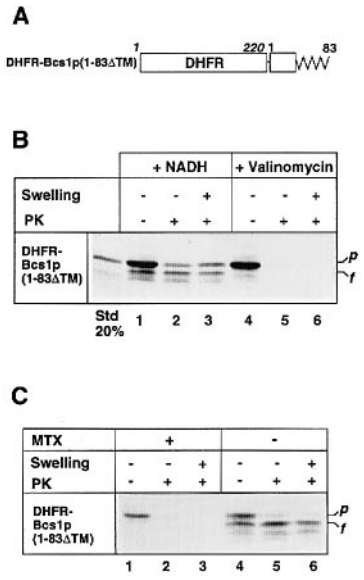

Fig. 2. A C-terminal targeting signal directs protein import into the mitochondrial matrix. (**A**) DHFR–Bcs1p(1–83ΔTM) fusion protein. (**B**) Radiolabelled DHFR–Bcs1p(1–83ΔTM) was imported into isolated mitochondria for 10 min at 25°C in the presence (+ NADH) or absence (+ Valinomycin) of a membrane potential. Mitochondria were reisolated and further treated as described in Figure 1B; Std 20%, 20% of the amount of radiolabelled protein added to each reaction; p, non-processed species; f, MPP-processed species. (**C**) Radiolabelled DHFR–Bcs1p(1–83ΔTM) was imported into mitochondria in the absence or presence of MTX. After import for 40 min at 25°C, mitochondria were reisolated and subjected to PK treatment as indicated; p, non-processed species; f, MPP-processed species.

A C-terminal targeting signal can direct a protein into mitochondria

Is C→N import dependent on the looped structure formed by the internal targeting signal or can a linear mitochondrial targeting signal at the C-terminus mediate import? We inactivated the looped internal targeting signal in DHFR–Bcs1p(1–83) by deleting the transmembrane domain which is essential for its formation and function (Fölsch *et al.*, 1996). The resulting chimera, DHFR–Bcs1p(1–83ΔTM), consists of DHFR at the N-terminus followed by a spacer of 45 amino acid residues and an α-helical positively charged import signal at the C-terminus (Figure 2A). Radiolabelled DHFR–Bcs1p(1–83ΔTM) was imported into the mitochondrial matrix in a Δψ-dependent fashion (Figure 2B). Following import, a large fraction of DHFR–Bcs1p(1–83ΔTM) underwent C-terminal processing, in a fashion similar to that observed for DHFR–Bcs1p(1–83). Import of DHFR–Bcs1p(1–83ΔTM) was inhibited when performed in the presence of methotrexate (Figure 2C). Thus, the translocation of an N-terminal DHFR moiety across the mitochondrial membranes can be inhibited by preventing its unfolding. Processing of the methotrexate-arrested DHFR–Bcs1p(1–83ΔTM) was observed after prolonged incubation, confirming that the maturation event occurred at the C-terminus of the protein (results not shown).

In conclusion, a chimeric protein bearing a C-terminal targeting signal can be imported backwards into the mitochondrial matrix in a linear fashion by embarking on a C→N translocation mechanism.

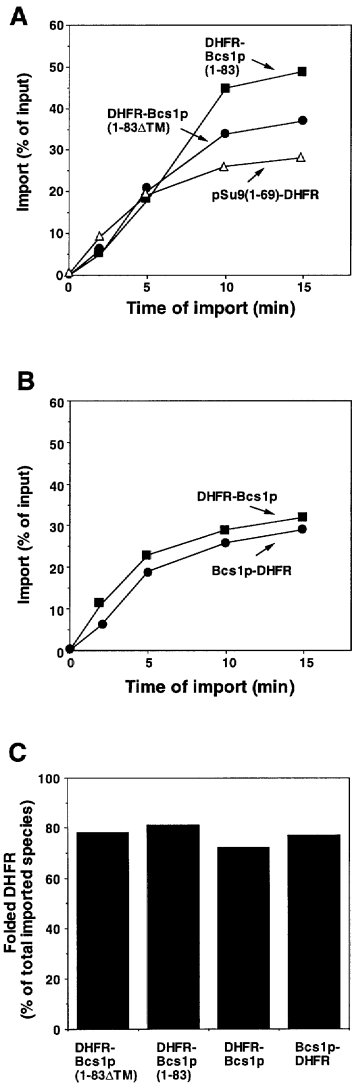

Fig. 3. Import in C→N direction is as efficient as the normal N→C import process. (**A** and **B**) Radiolabelled DHFR–Bcs1p(1–83), DHFR–Bcs1p(1–83ΔTM), pSu9(1–69)–DHFR (A) and DHFR–Bcs1p and Bcs1p–DHFR (B) were imported into isolated mitochondria at 25°C for the times indicated. Mitochondria were reisolated and were treated with PK. Samples were analysed by SDS–PAGE, fluorography and laser densitometry. Results are expressed as a percentage of the total radiolabelled material added to each import reaction (% of input). (**C**) Radiolabelled proteins were imported into isolated mitochondria, after which samples were cooled on ice and treated with trypsin. Mitochondria were reisolated and lysed in detergent, and folding of DHFR was assessed as previously described (Ostermann *et al.*, 1989). Results are expressed as a percentage of the imported DHFR-derived species which gave rise to folded DHFR domain.

Import of proteins in C→N direction is as efficient as the normal N→C import process

How efficient is this C→N backward-import process? We compared directly the import kinetics and efficiencies of DHFR–Bcs1p(1–83) and DHFR–Bcs1p(1–83ΔTM) with those of the N-terminal presequence-containing, matrix-targeted protein, pSu9(1–69)–DHFR (Figure 3A). Both DHFR–Bcs1p(1–83) and DHFR–Bcs1p(1–83ΔTM) were imported into mitochondria at a rate similar to that of pSu9(1–69)–DHFR. Furthermore, the import efficiencies of both of these DHFR–Bcs1p derivatives were high: 40–50% of the added material was imported (Figure 3A).

Thus, passage of proteins across the inner membrane

in a C→N direction most probably occurs by the same mechanism used by N-terminal targeted preproteins rather than by an alternative low-efficiency bypass route.

Can this C→N import pathway facilitate the import of proteins containing folded domains? We compared the kinetics and efficiency of import of DHFR–Bcs1p with that of Bcs1p–DHFR (a chimera where DHFR is placed at the C-terminus of Bcs1p; Fölsch *et al*., 1996). The DHFR domain in both cases was folded in the lysate as translocation of both preproteins can be arrested by the addition of methotrexate (Fölsch *et al*., 1996). These proteins were imported into isolated mitochondria with similar kinetics and efficiencies. Thus, the presence of a domain capable of folding prior to import, like DHFR, N- or C-terminal to an internal targeting signal, does not influence the import competence of the preprotein (Figure 3B). The pathway for C→N import can mediate the unfolding and translocation of folded domains on the outside of mitochondria, as efficiently as the normal N→C import process. Furthermore, import of DHFR–Bcs1p (1–83ΔTM) into isolated mitoplasts occurred as efficiently and with similar kinetics as import into isolated mitochondria (results not shown).

Does the DHFR moiety of these proteins refold upon import into the mitochondrial matrix? Following import and trypsin treatment, the imported proteins were solubilized by detergent, and the folded states of their DHFR moieties were assayed by resistance to added PK. The efficiency of refolding of the DHFR moiety following translocation into the mitochondrial matrix was not influenced by the direction of import: compare DHFR–Bcs1p(1–83ΔTM), DHFR–Bcs1p(1–83) and DHFR–Bcs1p with Bcs1p–DHFR (Figure 3C). On a separate note, gel electrophoresis indicated that the size of the DHFR protease-resistant moiety was similar in all cases (results not shown). This result again confirms that it was the C-terminus of the preprotein, rather than the N-terminus of DHFR–Bcs1p(1–83ΔTM) and DHFR–Bcs1p(1–83) which had undergone MPP processing following import.

A targeting signal derived from an authentic N-terminal presequence can target import of preproteins in a C→N fashion

Is the import direction C→N unique to proteins containing Bcs1p-derived mitochondrial targeting signals? We constructed a fusion protein in which a mitochondrial targeting signal (MTS) derived from an authentic N-terminal presequence was placed at the C-terminus of DHFR. We used a duplication of the residues 1–45 of the MTS of subunit 9 of the F_1F_0-ATPase of *Neurospora crassa*. The import into isolated mitochondria of the radiolabelled fusion protein DHFR–Su9(1–45) (1–45) was compared with that of the corresponding protein containing the same MTS at the N-terminus, pSu9(1–45) (1–45)–DHFR (Figure 4A). Both proteins were imported into the mitochondria, where they were located in the matrix (Figure 4A). Imported pSu9(1–45) (1–45)–DHFR was completely processed to its matured form by MPP. In contrast, the C-terminal presequence of DHFR–Su9(1–45) (1–45), was only partially processed following import (Figure 4A). In contrast to the DHFR–Bcs1p-derived proteins, the efficiency of the C-terminally targeted DHFR–Su9(1–45) (1–45) appeared lower than that of its N-terminally targeted counterpart. The last 15 amino acids (residues 30–45) do not have a strong mitochondrial targeting signal capacity (C.Ungermann and W.Neupert, unpublished observations). We therefore believe that this may account for the reduced import efficiency of the DHFR–Su9(1–45) (1–45).

The kinetics of processing of DHFR–Su9(1–45) (1–45) were markedly slower than those of its import (Figure 4B). This was in contrast to the pSu9(1–45) (1–45)–DHFR, whose kinetics of import and processing were indiscernible (Figure 4B). Processing in both cases was likely to be catalysed by MPP, since it was sensitive to the presence of metal chelators (results not shown). Therefore, MPP appears to display a preference for processing presequences located at the N-terminus of incoming proteins over those at the C-terminus.

When imported in the presence of methotrexate, both proteins remained accessible to exogenously added protease (Figure 4C). At the same time they were found in association with mt-Hsp70 in the mitochondrial matrix (Figure 4D). Thus, a C-terminally targeted protein can be arrested as a translocation intermediate spanning the outer and inner membranes. This occurs in a fashion similar to that which has been described previously for N-terminally targeted proteins (Schleyer and Neupert, 1985; Eilers and Schatz, 1986).

We then tested whether this C-terminal targeting signal was capable of mediating import of a protein into the mitochondrial matrix *in vivo*. Cytochrome oxidase subunit IV, an essential subunit of the cytochrome oxidase complex, is synthesized as a precursor protein containing an N-terminal cleavable presequence (pCOXIV). It is imported into the matrix and becomes processed to its mature-sized form (mCOXIV). We created a fusion protein whereby the preSu9(1–45) (1–45) targeting signal was added to the C-terminus of mCOXIV. The resulting construct, mCOXIV-Su9, was cloned into the yeast expression vector pVT102 and transformed into the Δ*coxIV* yeast strain, which is respiration incompetent (Figure 4E). Transformation with either a plasmid bearing the mCOXIV-Su9, or the original pCOXIV, led to complementation of the respiration-deficient phenotype of the Δ*coxIV* yeast strain.

We conclude, therefore, that a mitochondrial N-terminal presequence placed at the C-terminus of a passenger protein can target the import of preproteins in a C→N fashion, *in vitro* and *in vivo*. Furthermore, our data show that following import in a C→N manner *in vivo*, a passenger protein, COXIV, can fold and assemble correctly as a subunit of an oligomeric complex.

Import of protein in C→N direction occurs via the TIM17–23 machinery and is driven by mt-Hsp70

Do proteins imported in a C→N fashion use the same translocation machinery of the inner membrane as N-terminal-presequence-targeted proteins?

Translocation of preproteins across the inner membrane requires the ATP-dependent action of mt-Hsp70 (Cyr *et al*., 1993; Gambill *et al*., 1993; Stuart *et al*., 1994; Wachter *et al*., 1994). In ATP-depleted mitochondria the efficiency of import of DHFR–Bcs1p(1–83ΔTM) and the control protein Bcs1p were strongly reduced compared with matrix-ATP containing mitochondria (Figure 5A). A

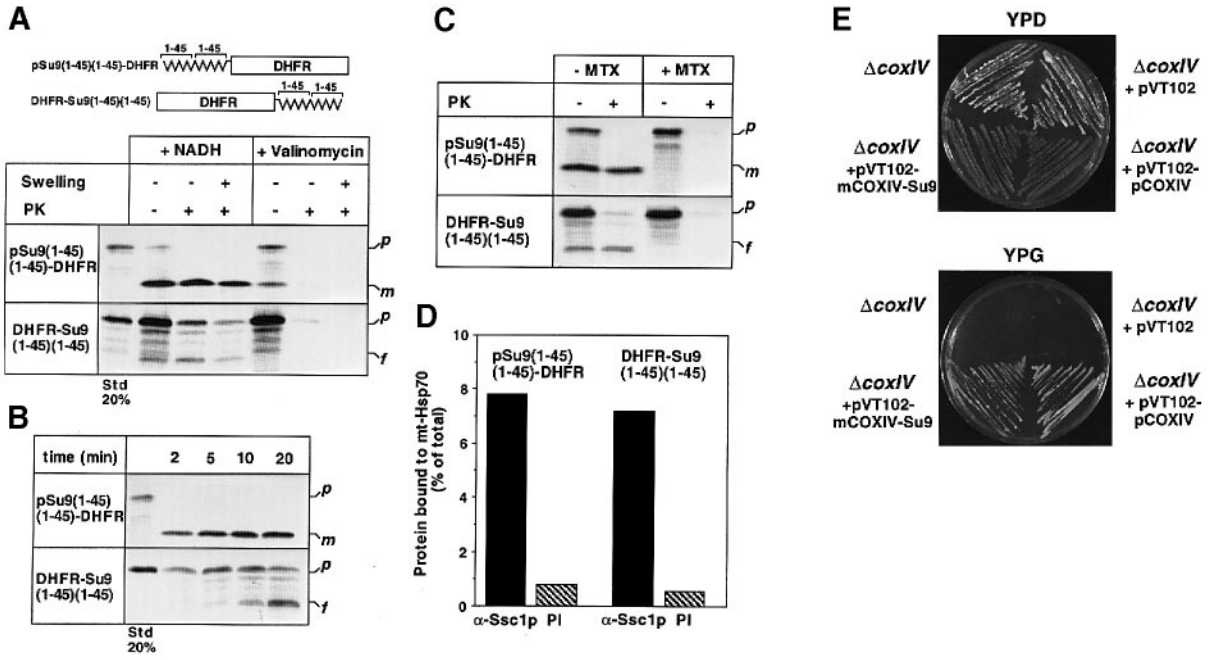

Fig. 4. An authentic N-terminal presequence can target import in a C→N fashion when present at the C-terminus of a passenger protein. Import of DHFR–Su9(1–45) (1–45) and pSu9(1–45) (1–45)–DHFR. (**A**) Radiolabelled proteins were imported into isolated mitochondria for 10 min at 25°C in the presence or absence of a membrane potential, as described in Figure 1B; p, non-processed species; m, MPP-processed pSu9(1–45) (1–45)–DHFR species; f, MPP-processed DHFR–Su9(1–45) (1–45) species; zig-zag lines denote the targeting sequence (amino acids 1–45, 1–45). DHFR, dihydrofolate reductase. (**B**) Radiolabelled proteins were imported at 25°C into mitochondria for the times indicated. Samples were subjected to hypotonic swelling and PK treatment, and subsequently analysed by SDS–PAGE and autoradiography. (**C**) Radiolabelled DHFR–Su9(1–45) (1–45) and pSu9(1–45) (1–45)–DHFR were imported into mitochondria in the absence or presence of MTX and NADPH. After import for 30 min at 25°C, mitochondria were reisolated and subjected to PK treatment, as indicated. All samples were analysed by SDS–PAGE, blotting onto nitrocellulose and autoradiography. (**D**) DHFR–Su9(1–45) (1–45) and pSu9(1–45) (1–45)–DHFR were accumulated as MTX-arrested translocation intermediates. Mitochondria were lysed in detergent buffer and specific association with mt-Hsp70 was determined by co-immunoprecipitation with antiserum raised against Ssc1p (α-Ssc1p) or preimmune serum (PI). (**E**) The Δ*coxIV* yeast strain transformed with either pVT102, pVT102-pCOXIV or pVT102-mCOXIV-Su9 plasmids (see Materials and methods) were streaked out on YPD (glucose) and YPG (glycerol) plate, as indicated, and were incubated for 3 days at 30°C. Rescue of the Δ*coxIV* petite phenotype by pCOXIV and mCOXIV-Su9 is observed through their ability to grow on YPG plates.

similar inhibition of import was observed when DHFR–Bcs1p(1–83ΔTM) was imported into mitochondria from the mutant *ssc1-3*, which harbours a temperature sensitive mt-Hsp70 (Figure 5B).

Which of the two established TIM complexes, Tim17–23 or Tim22–54 (Sirrenberg *et al*., 1996; Kerscher *et al*., 1997), facilitates the C→N translocation of C-terminally targeted proteins? DHFR–Bcs1p(1–83ΔTM) was imported into mitochondria isolated from a yeast strain in which the expression levels of Tim22 had been down-regulated (Tim22↓). These Tim22↓ mitochondria displayed an impaired ability to import the ADP/ATP carrier (AAC) (Figure 5B; Sirrenberg *et al*., 1996). The efficiency of import of DHFR–Bcs1p (1–83ΔTM), like that of the N-terminally targeted pSu9 (1–79)–DHFR precursor, was unaffected in these Tim22↓ mitochondria, indicating that Tim22 does not play a role in this C→N import process. In contrast, mitochondria harbouring a modified form of Tim23, Tim23(fs), display a strongly reduced capacity to import both N-terminally targeted proteins, pSu9(1–79)–DHFR (Figure 5B; Sirrenberg *et al*., 1996) and the C-terminally targeted DHFR–Bcs1p(1–83ΔTM). The import of AAC was unaffected in these mutant mitochondria, as reported previously (Figure 5B; Sirrenberg *et al*., 1996).

In conclusion, the process of C→N import, directed either by an internal targeting signal or by a C-terminal targeting signal, displays the same requirement for mt-Hsp70 and Tim23 as N-terminally targeted precursor proteins.

Discussion

In the present study we addressed the directionality of the import mechanism across the inner membrane. Initially we investigated the import of proteins with the looped internal targeting signal of the Bcs1p protein. This internal signal mediates the translocation across the mitochondrial inner membrane of segments of the polypeptide chain both N- and C-terminal to it. The TIM17–23 complex has been proposed to form dimers (Bauer *et al*., 1996), and therefore each side of the dimer might facilitate the translocation of one side of the looped internal targeting signal. The mt-Hsp70-assisted translocase appears to display no specificity for the directionality of the incoming polypeptide chain. It operates in a C→N direction on the N-terminal side of the looped signals and in an N→C direction on the opposite side. In confirmation of this, mitochondrial targeting signals placed at the C-terminus of a passenger protein enabled the 'backwards' import of the protein in a linear, C→N fashion across the inner membrane into the mitochondrial matrix, followed by processing of the targeting signal. This backwards import occured with comparable rates and efficiencies as normal,

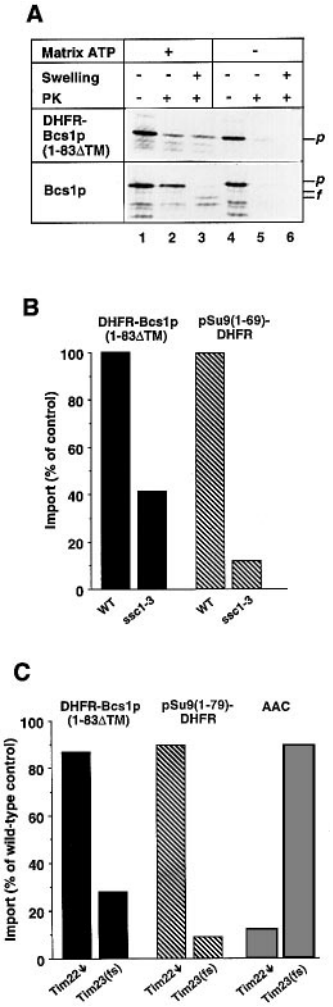

Fig. 5. Import in the C→N direction occurs via TIM17–23 machinery and is driven by mt-Hsp70. (**A**) Isolated mitochondria were incubated in import buffer and were either depleted of matrix ATP (–ATP) or mock-treated (+ATP), as described previously (Stuart *et al*., 1994). Radiolabelled preproteins were imported for 5 min at 25°C. After import, mitochondria were reisolated, and either mock-treated or PK-treated under non-swelling or swelling conditions, as indicated. Opening of the intermembrane space was >95% efficient, while the integrity of the inner membrane was not perturbed (data not shown); p, imported species; f, the mobility of the fragments generated upon PK treatment of the mitoplasts. (**B**) Radiolabelled preproteins were imported into isolated *ssc1-3* mitochondria (*ssc1-3*) or isogenic wild-type (WT) following their preincubation at the non-permissive temperature of 37°C. Mitochondria were reisolated and were treated with PK. Samples were analysed by SDS–PAGE, fluorography and laser densitometry. Results are expressed as a percentage of import achieved in the wild-type control mitochondria (% of control). (**C**) Radiolabelled precursors DHFR–Bcs1p(1–83ΔTM), pSu9(1–79)–DHFR and AAC were imported into wild-type, Tim22↓ and Tim23(fs) mitochondria, as indicated. Samples were treated with PK, after which they were analysed by SDS–PAGE, fluorography and laser densitometry. Imported protein is expressed as a percentage of import into wild-type mitochondria.

forward N→C import. Furthermore, this novel import pathway facilitates the unfolding of folded domains in a manner similar to the unfolding of preproteins with N-terminal signals.

In conclusion, the TIM17–23 machinery of the inner membrane, together with mt-Hsp70 in the matrix, can effectively mediate the import of preproteins, irrespective of their orientation during translocation across the mitochondrial membranes. This observation implies that the peptide binding sites of mt-Hsp70 display no specificity for polarity, binding incoming polypeptides in both an N→C and C→N fashion in a productive and equally efficient manner.

If the 'backwards' C→N terminal import is so efficient, why do the authentic presequence-targeted mitochondrial proteins identified to date contain N-terminal rather than C-terminal targeting signals? In contrast to components of the inner-membrane translocase and mt-Hsp70, MPP clearly displayed a preference for presequences imported in an N→C fashion. When present at the C-terminus of a protein, kinetics of processing were significantly slower than those of import. This was in marked contrast to the fate of an N-terminal presequence. In this case the kinetics of processing paralleled those of import, indicating that maturation occurs in a co-translocational manner. By becoming processed during import, the incoming protein is ensured of efficient processing prior to its refolding in the matrix. Retardation of processing caused by having the presequence present at the C-terminus of the protein may be adverse to ensuing events such as folding, assembly into oligomeric structures and in some cases export from the matrix.

Import of nuclear encoded proteins into mitochondria can occur in a post-translational manner both *in vitro* and *in vivo* (see Neupert, 1997, for discussion). The presence of an N-terminal signal would be advantageous, as both targeting and translocation of certain proteins may be initiated prior to their completion of synthesis. This might reduce the possibility of mistargeting to other cellular compartments, a process which might be favoured if the targeting signal were present at the C-terminus of the protein. Placement of the targeting signal to the C-terminus may delay the kinetics of the import reaction and thereby could be argued to increase the possibility for folding and/or aggregation.

Although nature clearly favours the presence of N-terminal, rather than C-terminal mitochondrial targeting sequences, we suspect that some as yet unidentified mitochondrial proteins may exist with C-terminal import signals. Bearing in mind the efficiency of the C-terminal targeting pathway, the possibility of such a pathway existing *in vivo* cannot be excluded, especially if the C-terminal signal turns out to be a non-cleavable one. The confirmation of such a targeting mechanism *in vivo* of authentic mitochondrial proteins would have important consequences for the future identification of putative mitochondrial proteins from genome-sequencing data.

Materials and methods

Cloning of DHFR–Bcs1p(1–83), DHFR–Bcs1p(1–83ΔTM), pSu9(1–45) (1–45)–DHFR and DHFR–Su9(1–45) (1–45)

The DHFR–Bcs1p(1–83) fusion protein was cloned by PCR using the full-length DHFR–Bcs1p clone (Fölsch *et al*., 1996) as a template. The PCR products were then cloned using *Eco*RI–*Hin*dIII in the *in vitro* transcription vector pGEM4 under the control of the Sp6 RNA polymerase.

Deletion of the membrane anchor [DHFR–Bcs1p(1–83ΔTM)] was achieved by amplifying the DHFR–Bcs1p(1–43) fragment by PCR using the DHFR–Bcs1p (Fölsch *et al*., 1996) as template, the N-terminal primer as above and the following C-terminal primer: 5′-CCCCGGATCCGCCAACTAGTTTTGAAAG-3′. The PCR product was cloned as a *Bam*HI–*Bam*HI fragment in front of the Bcs1p(Δ65) construct (Fölsch

et al., 1996) to result in a DHFR–Bcs1p(TM) clone. In a second PCR reaction this clone was used as a template. The N- and C-terminal primers used, as well as the subcloning, were as described above for the DHFR–Bcs1p(1–83) clone.

The DHFR moiety used in the pSu9(1–45) (1–45)–DHFR and DHFR–Su9(1–45) (1–45) was amplified by PCR as a *Bam*HI–*Hin*dIII fragment, whereby an in-frame *Bgl*II site was introduced prior to the stop codon. This PCR product was cloned as a *Bam*HI–*Hin*dIII fragment into a pGEM4 vector. The initial 45 amino acids of the presequence of subunit 9 of the F_1F_0-ATPase of Neurospora crassa were amplified as a *Bam*HI–*Bgl*II fragment. This was cloned either into the *Bam*HI site prior to the cloned DHFR to yield pSu9(1–45)–DHFR or into the *Bgl*II site prior to the STOP codon of DHFR, to yield DHFR–Su9(1–45). The resulting plasmids were linearized once again with *Bam*HI (in the case of pSu9 (1–45)–DHFR) or *Bgl*II (in the case of DHFR–Su9(1–45) and the Su9 PCR fragment was cloned in again, to result in pSu9(1–45) (1–45)–DHFR and DHFR–Su9(1–45) (1–45), respectively.

Cloning and expression of pCOXIV and mCOXIV-Su9

The DNA encoding pCOXIV was amplified from yeast genomic DNA by PCR and was cloned as a *Bam*HI–*Hin*dIII fragment directly into the yeast expression vector pVT102 (2μ, URA3), under the control of the ADH1 promoter (Vernet *et al*., 1987). Mature-size COXIV (codons 25–155) (mCOXIV) was amplified by PCR without the stop codon, whereby the restriction sites *Eco*RI and *Bam*HI were introduced at the 5′ end and *Bgl*II at the 3′ end of the fragment. The resulting fragment was cloned as a *Eco*RI–*Bgl*II insert into pGEM4–DHFR–Su9(1–45) (1–45), which had been cut previously with *Eco*RI–*Bgl*II to remove the DHFR. The resulting construct, mCOXIV–Su9, was cloned as a *Bam*HI–*Hin*dIII fragment into pVT102. The yeast Δ*coxIV* strain (W303–1A, *Mat***a**, *leu2, ura3, ade2, can1*, COXIV::TRP1) was transformed with either pVT102, pVT102-pCOXIV or pVT102-mCOXIV-Su9 plasmids, and URA + clones were selected. These transformants, together with the original Δ*coxIV* strain, were streaked out on YPD (glucose-containing) or YPG (glycerol-containing) plates and incubated at 30°C. Complementation of the Δ*coxIV* respiratory petite phenotype was tested by assessing its ability to grow on glycerol (non-fermentable carbon source).

Isolation of mitochondria and protein import

Saccharomyces cerevisiae wild-type strain (D273–10B) was grown in lactate medium at 30°C (Herrmann *et al*., 1994), whereas the temperature-sensitive mutant of the SSC1 gene product, mt-Hsp70, termed *ssc1-3* (PK83) and its isogenic wild-type strain (PK82) (Gambill *et al*., 1993) were grown at 24°C. The Tim22(Gal 10) strain (Sirrenberg *et al*., 1996) was grown at 30°C in minimal medium supplemented with 2% lactate, 0.1% glucose and either in the presence (Tim22↑) or absence (Tim22↓) of 1% galactose for five generations. The Tim23(fs) strain and its isogenic wild-type strain (Sirrenberg *et al*., 1996) were grown at 24°C in minimal medium with 2% lactate, 0.1% glucose and 1% galactose. Cells were harvested at an OD_{578} of ~1 and mitochondria were isolated as described previously (Herrmann *et al*., 1994). Isolated mitochondria were resuspended in 250 mM sucrose, 10 mM MOPS pH 7.2 and 1 mM EDTA (SEM-buffer) at a protein concentration of 10 mg/ml.

Bcs1p–DHFR and pSu9–DHFR- (Ungermann *et al*., 1994) derived fusion proteins were synthesized in rabbit reticulocyte lysate (Promega Corp., USA) in the presence of [^{35}S]methionine, as described previously (Pelham and Jackson, 1976).

Import into mitochondria was performed as described before, in buffer A: 3% (w/v) bovine serum albumin (BSA), 50 mM HEPES (pH 7.2), 0.5 M sorbitol, 80 mM KCl, 10 mM MgOAc, 2 mM K-phosphate, 2.5 mM EDTA, 2.5 mM $MnCl_2$. Import mixtures usually contained 4 mM NADH, 2 mM ATP, 0.2 mg protein/ml mitochondria and 2% (v/v) reticulocyte lysate (Stuart *et al*., 1994). Import was performed at 25°C for the times indicated. Following import, samples were treated with PK under non-swelling or swelling conditions.

Hypotonic swelling of mitochondria was performed as follows: after import, mitochondria were reisolated by centrifugation (Sigma, rotor 12153, 5 min 16 300 *g*, 2°C), resuspended in buffer A at a concentration of 50 μg/100 μl and then diluted 10-fold in 20 mM HEPES (pH 7.2), in the presence of PK (100 μg/ml). Control mitochondria (non-swelling conditions) were diluted to the same extent in SH buffer (0.6 M sorbitol, 20 mM HEPES pH 7.2) and also subjected to PK treatment, where indicated. Samples were kept on ice for 30 min and PMSF (2 mM) was added. Mitoplasts/mitochondria were reisolated by centrifugation and washed once with SH-buffer supplemented with 80 mM KCl and 0.3 mM PMSF, and then were lysed directly in SDS sample buffer. Samples were analysed by SDS–PAGE and blotting onto nitrocellulose. The efficiency of swelling was assessed following immunodecoration of the blot with antisera against endogenous CCPO (soluble intermembrane space protein) and Mge1p (matrix-located protein).

Miscellaneous

The following procedures were performed according to published methods: protein determination (Bradford, 1976), SDS–PAGE (Laemmli, 1970) and import of preproteins in the presence of methotrexate (Fölsch *et al*., 1996). The detection of proteins after blotting onto nitrocellulose was performed using the ECL detection system (Amersham).

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