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Conservative sorting of F_0-ATPase subunit 9: export from matrix requires ΔpH across inner membrane and matrix ATP

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In an attempt to understand the mechanisms of sorting of mitochondrial inner membrane proteins, we have analysed the import of subunit 9 of the mitochondrial F_1F_0-ATPase (Su9) from *Neurospora crassa*, an integral inner membrane protein. A chimeric protein was used consisting of the presequence and the first transmembrane domain of Su9 fused to mouse dihydrofolate reductase (preSu9(1–112)–DHFR). This protein attains the correct topology across the inner membrane (N_{out}–C_{in}) following import. The transmembrane domain becomes first completely imported into the matrix, where after processing of the presequence, it mediates membrane insertion and export of the N-terminal tail. Import and export steps can be experimentally dissected into two distinct events. Translocation of the N-terminal hydrophilic tail out of the matrix was blocked when the presequence was not processed, indicating an important role of the sequences and charges flanking the hydrophobic domain. Furthermore, export was supported by a ΔpH and required matrix ATP hydrolysis. Thus the hydrophobic transmembrane domain operates as a membrane insertion signal and not as a stop–transfer signal. Our findings suggest that several aspects of this sorting process have been conserved from their prokaryotic ancestors.

Key words: F_1F_0-ATPase subunit 9/intramitochondrial sorting/membrane insertion/mitochondria/*Saccharomyces cerevisiae*

Introduction

A central theme in mitochondrial biogenesis is the mechanism of sorting of nuclear encoded preproteins to the four mitochondrial subcompartments, namely the outer membrane, the inner membrane, the intermembrane space and the matrix (Glick *et al.*, 1992; Stuart *et al.*, 1995). Both the outer and inner membrane contain translocation machineries which mediate the specific translocation of mitochondrial precursor proteins across these membranes (Maarse *et al.*, 1992; Scherer *et al.*, 1992; Blom *et al.*, 1993; Emtage and Jensen, 1993; Horst *et al.*, 1993; Mayer *et al.*, 1993, 1995; Ryan and Jensen, 1993; Ryan *et al.*, 1994; Schneider *et al.*, 1994). Proteins targeted to the matrix reach this compartment by their movement through both of these machineries, while proteins destined for other compartments are sorted selectively at different stages of the import process. Of particular interest is the sorting and assembly of integral inner membrane proteins. The hydrophobic transmembrane domains of such proteins may act as topogenic signals in analogy with other membrane systems, but it is far from clear how these signals function. In principle one can distinguish two possible modes of their operation, either as stop–transfer signals or as membrane insertion signals (Blobel, 1980; Hartl and Neupert, 1990; High and Dobberstein, 1992). A stop–transfer signal would cause an arrest of the protein at the level of the inner membrane import machinery and thereby prevent further translocation. Proteins would undergo lateral sorting and for those where the transmembrane domain forms an integral part of the final protein its N-terminus would therefore be present in the matrix (Miller and Cumsky, 1993). As this clearly is not always the situation and many proteins contain their N-terminus in the intermembrane space, alternative mechanisms of sorting must exist.

Here we have addressed the sorting of subunit 9 of the F_0-ATPase (Su9), an integral inner membrane protein. It is a member of that class of nuclear-encoded mitochondrial proteins which are evolutionarily derived from a prokaryotic ancestor in accordance with the endosymbiotic hypothesis. In yeast, Su9 is a mitochondrially encoded protein. In contrast, in *Neurospora crassa* and higher eukaryotes it is synthesized in the cytosol as a precursor (preSu9). PreSu9 contains a long N-terminal presequence consisting of 66 amino acids in the case of *N.crassa*. This presequence is cleaved in two steps by the mitochondrial processing peptidase (MPP) at positions 35 and 66. Mature Su9 contains two hydrophobic stretches, each of sufficient length to span the inner membrane once. A comparison with the homologous ATPase subunit c of bacteria suggests that both N- and C-termini are located in the intermembrane space (N_{out}–C_{out}) (Fillingame, 1981; Sebald and Hoppe, 1981; Mao *et al.*, 1982). A preliminary study of the import of this protein has confirmed this for the C-terminus of the mitochondrial protein (Mahlke *et al.*, 1990).

In the present study we have analysed the import and sorting of a fusion protein consisting of the first transmembrane domain of Su9 fused to mouse cytosolic dihydrofolate reductase (DHFR). We present evidence that the membrane anchor of this protein serves as an insertion signal and not as a stop–transfer signal. We show that this protein is completely imported across both outer and inner membrane translocation channels, despite its hydrophobicity. Following accumulation in the matrix, it then undergoes export back across the inner membrane. Several aspects of this sorting process resemble protein export in prokaryotes. We therefore propose that this mitochondrial sorting pathway has been conserved through evolution from its prokaryotic ancestors.

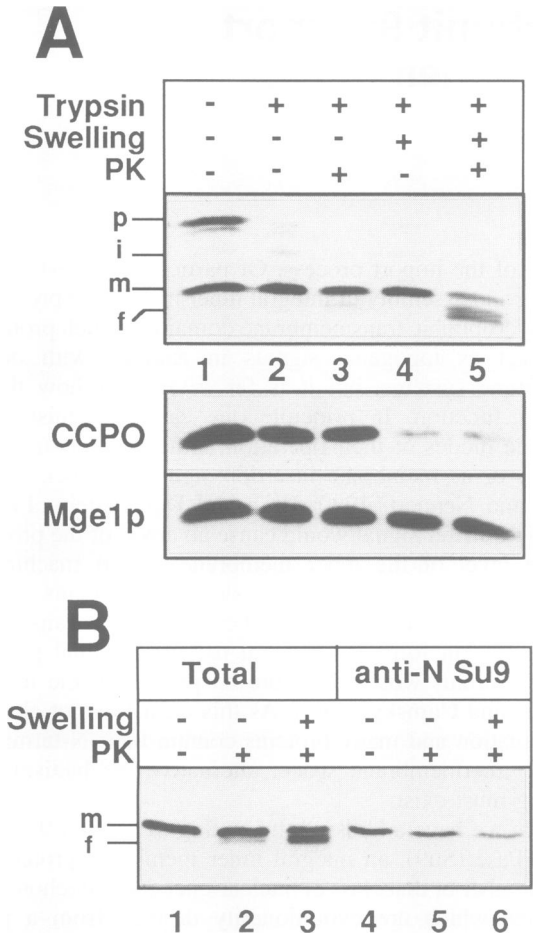

Fig. 1. Import and sorting of preSu9(1–112)–DHFR. [^{35}S]methionine-labelled preSu9(1–112)–DHFR was synthesized in reticulocyte lysate and imported into mitochondria at 25°C for 15 min. Trypsin-treated mitochondria were re-isolated and where indicated were converted to mitoplasts by swelling mitochondria in low-osmotic strength buffer, either in the absence or presence of proteinase K. (**A**) Samples were analysed by SDS–PAGE and fluorography. Forms of preSu9(1–112)–DHFR: p, precursor; i, intermediate; m, mature; f, fragment. Cytochrome *c* peroxidase (CCPO) and Mge1p were immunoblotted as controls for the opening of the intermembrane space and the matrix. (**B**) Following import, mitochondria were lysed in Triton X-100 buffer and samples were immunoprecipitated with an antibody raised against a peptide corresponding to the extreme N-terminus of *N.crassa* Su9 (anti-N Su9).

Results

PreSu9(1–112)–DHFR is correctly sorted in yeast mitochondria

In order to analyse sorting of Su9 across the inner membrane we have used a fusion protein, preSu9(1–112)–DHFR (Ungermann *et al.*, 1994). This protein consists of the presequence of Su9 and the first half of the mature part of the protein (comprising the first transmembrane domain) and of the DHFR at the C-terminus. The predicted topology of this protein is N_{out}–C_{in}, the N-terminal tail of ~14 amino acids being exposed to the intermembrane space leaving the C-terminal DHFR in the matrix.

Radiolabelled preSu9(1–112)–DHFR was imported into isolated mitochondria where it became efficiently processed to its mature-size form, mSu9(1-112)–DHFR, and inaccessible to exogenously added trypsin (Figure 1A, compare lanes 1 and 2). The protein attained the correct topology as revealed by subfractionation of mitochondria. Hypotonic swelling in the presence of proteinase K resulted in the removal of the short N-terminal tail, thus generating a slightly shorter protease-protected fragment [f-Su9(1–112)–DHFR] (Figure 1A, lane 5). This fragment resulted from removal of the extreme N-terminus of the protein as it could be no longer immunoprecipitated with an antibody raised against a peptide corresponding to the extreme N-terminus of the mature Su9 (Figure 1B, lane 6). Radiosequencing also confirmed that this fragment was generated following removal of the N-terminal 13 amino acids from the protein (results not shown).

Thus it appears that following MPP cleavage in the matrix the N-terminus of the protein becomes re-translocated across the inner membrane to the intermembrane space.

The presence of hydrophobic domain in preSu9(1–112)–DHFR renders mt-Hsp70 dispensable for presequence stabilization during import

The requirement of matrix ATP/mt-Hsp70 for the import of matrix-targeted precursors has been well documented recently. Mt-Hsp70 action is necessary for both stabilization of the presequence in the matrix and for completion of translocation of the polypeptide chain across the inner membrane (Cyr *et al.*, 1993; Stuart *et al.*, 1994a,b; Ungermann *et al.*, 1994; Wachter *et al.*, 1994). Like the import of the matrix-targeted protein preSu9(1–69)–DHFR (the transmembrane domain being absent), the import of preSu9(1–112)–DHFR was dependent on mt-Hsp70 action; import into mitochondria which had been depleted of matrix ATP was greatly inhibited (Figure 2A and B, compare lanes 5 and 6 with lanes 7 and 8). The processing of preSu9(1–112)–DHFR by MPP was unaffected by the matrix ATP depletion, in contrast to the processing of preSu9(1–69)–DHFR (Figure 2A and B, compare lanes 1 and 2 with lanes 3 and 4) and other matrix-targeted proteins (results not shown; Cyr *et al.*, 1993). Absence of mt-Hsp70 action therefore leads to accumulation of the preSu9(1–112)–DHFR as an import intermediate processed by MPP and largely accessible to exogenously added proteases. The fraction of the preSu9(1–112)–DHFR which had been imported was accessible to proteases when the outer membrane was disrupted, suggesting that it had accumulated spanning the inner membrane (results not shown). Thus, the presence of the hydrophobic domain in preSu9(1–112)–DHFR renders the incoming precursor independent of mt-Hsp70 for presequence cleavage. This appears to be the result of an interaction of the hydrophobic domain with the import machinery of the inner membrane or a component in the matrix.

The import and export steps of preSu9(1–112)–DHFR are two distinct events

We asked whether we could experimentally dissect the export reaction from the import process, to enable an independent study. Kinetic analysis showed that these two steps can be temporally separated (Figure 3A). After 2 min of import the imported species was located entirely in the matrix (Figure 3A, lane 4). Export resulting in the exposure of a protease-accessible domain in the intermembrane space was observed only at later time

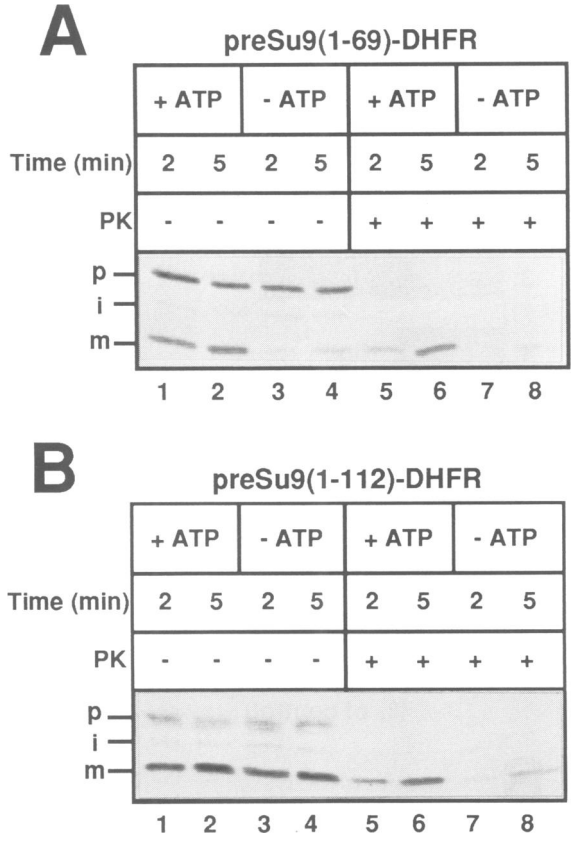

Fig. 2. Import of preSu9(1–112)–DHFR into matrix ATP-depleted mitochondria. PreSu9(1–69)–DHFR (**A**) and preSu9(1–112)–DHFR (**B**) were imported into either mock-treated (+ATP) or matrix ATP-depleted (–ATP) mitochondria (Stuart *et al.*, 1994b) in the presence of external ATP for either 2 or 5 min at 25°C. Samples were divided and either treated with proteinase K (PK, 40 μg/ml) or not as indicated. Analysis of the samples was as in Figure 1.

points (Figure 3A, lanes 8 and 12). The mSu9(1–112)–DHFR which had accumulated in the matrix after early time points of import was a productive sorting intermediate as it could be further chased at 25°C (Figure 3B). This resulted in the translocation of the N-terminus across the inner membrane (Figure 3B, lane 7). Upon incubation at 0°C the imported species remained in the matrix, thus demonstrating that the export event was temperature-sensitive (Figure 3B, lane 4).

Maintenance of the presequence prevents export of preSu9(1–112)–DHFR from the matrix

In order to address whether removal of the positively-charged presequence was necessary before export, preSu9(1–112)–DHFR was imported in the presence of chelating agents EDTA/*o*-phenanthroline (EDTA/*o*-phe), which inhibit the metal-dependent MPP activity (Schmidt *et al.*, 1984). Under these conditions preSu9(1–112)–DHFR was accumulated in the matrix of mitochondria (Figure 4, lanes 2 and 4). When MPP was reactivated by the addition of Mg^{2+}/Mn^{2+}, both efficient processing (Figure 4, lane 8) and export of the N-terminus into the intermembrane space occurred (Figure 4, lane 10). Maintenance of MPP inhibition during the second reaction resulted in a continued block of the export event (Figure 4, lane 7). Thus the presence of the uncleaved presequence prevented the export of the N-terminus.

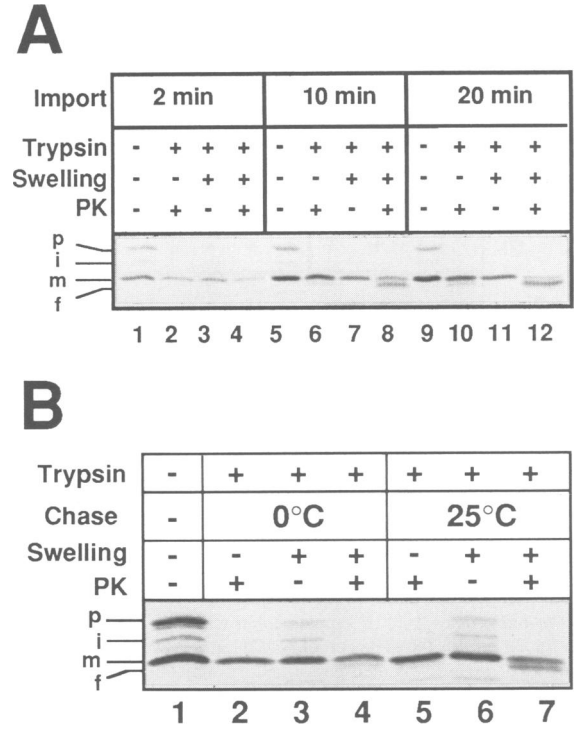

Fig. 3. Separation of import into the matrix from insertion into the inner membrane. (**A**) Kinetics of the import and export process. Radiolabelled preSu9(1–112)–DHFR was imported into mitochondria for either 2, 10 or 20 min. Samples were treated with trypsin where indicated, mitochondria were re-isolated and mitoplasts generated in the presence of proteinase K (PK) to assess export by the formation of the f-form. Note: the small amount of f-form present in lanes 6 and 10 was due to minor damage of the outer membrane during the protease treatment, as confirmed by immunoblotting of endogenous intermembrane space marker proteins (results not shown). (**B**) Chase of accumulated preSu9(1–112)–DHFR from the matrix into the inner membrane. Import of preSu9(1–112)–DHFR was performed for 2 min at 25°C, after which the sample was treated with trypsin. Mitochondria were re-isolated and resuspended in fresh import buffer and further incubated in a chase reaction for 15 min at either 0 or 25°C, as indicated. Mitoplasts were generated (+ swelling) in the absence or presence of proteinase K.

Export of preSu9(1–112)–DHFR from matrix requires ΔpH and matrix ATP hydrolysis

Does the export of the N-terminus of preSu9(1–112)–DHFR from the matrix require an energized inner membrane? PreSu9(1–112)–DHFR was accumulated in the mitochondrial matrix in the presence of EDTA/*o*-phe and chased following the addition of divalent cations either in the absence or presence of inhibitors of the membrane potential. In the absence of added inhibitors, efficient export of the N-terminus was observed (Figure 5A, lane 1). This efficiency was enhanced to a small extent following the addition of NADH (Figure 5A, lane 2). Export was slightly affected by valinomycin (K^+ ionophore, dissipates $\Delta\Psi$) (Figure 5A, lane 3), at concentrations which completely prevent import across the inner membrane. In contrast, export was strongly inhibited by nigericin (facilitates exchange of K^+ for H^+, dissipates the ΔpH) (Figure 5A, lane 4, 72% inhibition) or CCCP (protonophore, dissipates ΔpH and therefore $\Delta\Psi$) (Figure 5A, lane 6, 80% inhibition). The degree of inhibition by nigericin was higher in the presence of valinomycin (Figure 5A, lane 5, 80% inhibition).

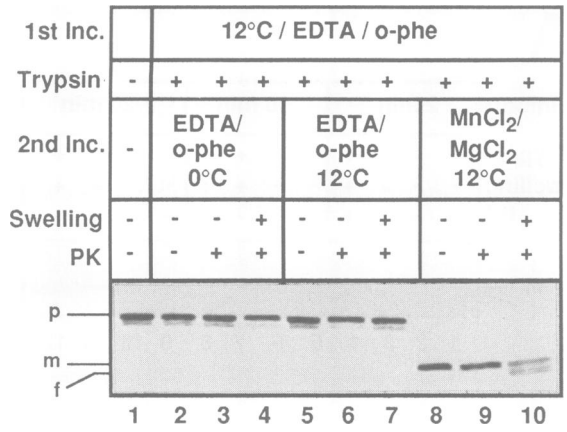

Fig. 4. Prevention of export by inhibition of presequence cleavage. PreSu9(1–112)–DHFR was imported into mitochondria for 30 min at 12°C in the presence of 10 mM EDTA and 2 mM *o*-phenanthroline (*o*-phe). The sample was treated with trypsin. Mitochondria were resuspended in fresh import buffer and divided into three aliquots. These were subjected to a second incubation at either 0°C (lanes 2–4) or 12°C (lanes 5–10), either in the presence of continuing MPP block (EDTA/*o*-phe) (lanes 2–7) or supplemented with Mg^{2+}/Mn^{2+} (lanes 8–10) for 30 min in the presence of NADH and ATP. Localization of the imported species was then performed by subjecting the mitochondria to hypotonic swelling treatment in the presence of proteinase K.

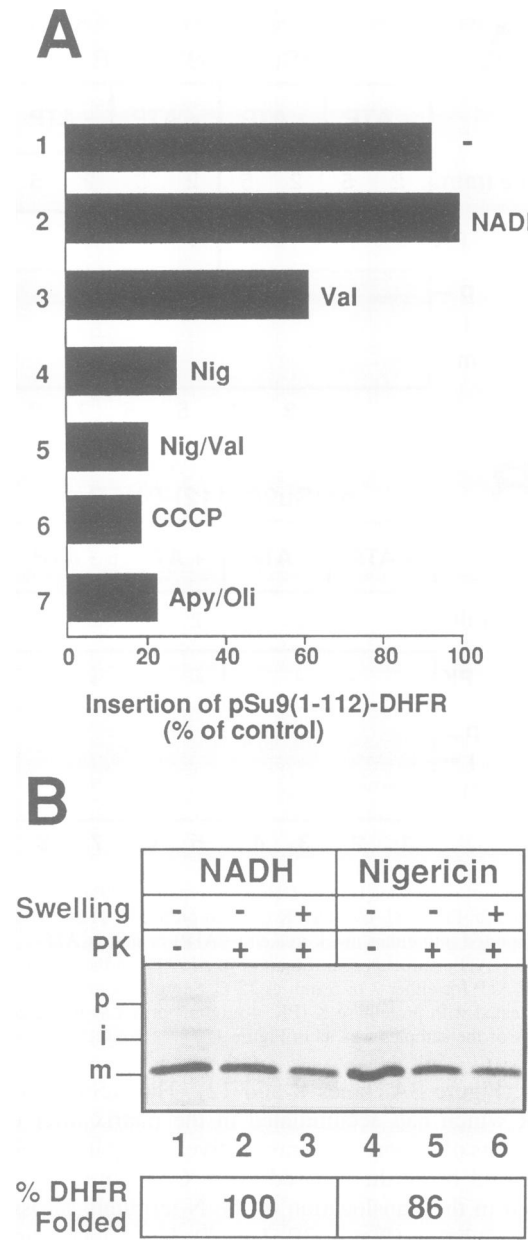

Fig. 5. Energetic requirements of the export step: (**A**) PreSu9(1–112)–DHFR was accumulated in the matrix in the presence of EDTA/*o*-phe as described in Figure 4. Chase in the presence of Mg^{2+}/Mn^{2+} was at 12°C for 30 min in the presence of 0.1% (v/v) ethanol (inhibitor solvent) and the following additions: no further additions (lane 1), 2 mM NADH (lane 2), 1 μM valinomycin (lane 3), 10 μM nigericin (lane 4), 10 μM nigericin plus 1 μM valinomycin (lane 5), 100 μM CCCP (lane 6), 40 U/ml apyrase plus 20 μM oligomycin (lane 7). Following chase, samples were subjected to osmotic swelling in the presence of proteinase K. Efficiency of export is expressed as percentage of control (+NADH). (**B**) PreSu9(1–112)–DHFR was imported into mitochondria for 2.5 min at 25°C, either in the presence of 2 mM NADH (lanes 1–3) or 10 μM nigericin (lanes 4–6). Samples were divided and mitochondria were re-isolated and where indicated were converted to mitoplasts by swelling mitochondria in low-osmotic strength buffer, either in the absence or presence of proteinase K. Mitochondria from the other set of aliquots were lysed in detergent and folding of DHFR was assessed (Ostermann *et al.*, 1989). Results are expressed as percentage of control (+NADH).

The following observations support the notion that the inhibition by nigericin was not due to a secondary effect on matrix ATP synthesis: (i) import of preSu9(1–112)–DHFR into the mitochondrial matrix was not inhibited in the presence of nigericin, thus the matrix ATP levels were sufficiently high to allow ATP-dependent mt-Hsp70 action (Figure 5B); (ii) the folding of the DHFR domain in the matrix, an ATP-dependent event, was also not inhibited in nigericin-treated mitochondria (Figure 5B); (iii) the nigericin inhibition could not be overcome by the addition of α-ketoglutarate/succinate/malate, which promote efficient matrix ATP synthesis (Wachter *et al.*, 1994) (results not shown). Furthermore, the addition of very high amounts of ATP could only partially overcome the inhibitory effect of nigericin on the export process (results not shown).

To test directly whether in addition to ΔpH, ATP is required for the export process, matrix accumulated preSu9(1–112)–DHFR was chased in the presence of low matrix ATP levels following reactivation of MPP by the addition of Mg^{2+}/Mn^{2+} (Figure 5A, lane 7). Export was inhibited when matrix ATP was limiting despite the presence of a $\Delta\mu H^+$ (presence of NADH).

PreSu9(1–112)–DHFR accumulated in the matrix was present in a complex with mt-Hsp70 as shown by co-immunoprecipitation analysis (Figure 6, lanes 1–3). This complex remained stable throughout the 30 min chase period. The DHFR domain was tightly folded (results not shown), suggesting that the interaction of mt-Hsp70 occurred with the Su9 part of the protein. In the presence of ATP, chase of the mature-sized species to the exported form was accompanied by release from mt-Hsp70. The level of Su9(1–112)–DHFR associated with mt-Hsp70 decreased by 75% over the 30 min chase period (Figure 6, lanes 4–6). Under matrix ATP-depletion conditions this release step was prevented, as was the export step, as only a 30% reduction in the species complexed to mt-Hsp70 was observed (Figure 6, lanes 10–12). When the chase was performed in the presence of nigericin, mSu9(1–112)–DHFR

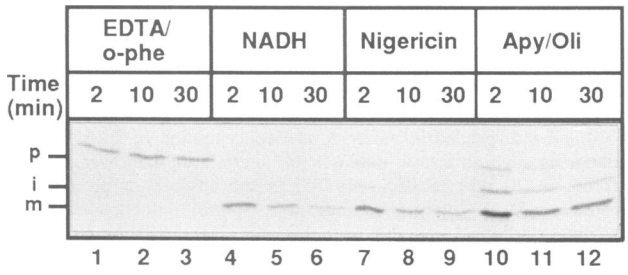

Fig. 6. Release from mt-Hsp70 precedes export step. Radiolabelled preSu9(1–112)–DHFR was imported into mitochondria as described in Figure 4. Following trypsin treatment, samples were kept on ice (lanes 1–3) chased in the presence of NADH (lanes 4–6), of nigericin (lanes 7–9) or of apyrase/oligomycin (lanes 10–12) as described in Figure 5A, for times indicated. Samples were returned to ice, treated with oligomycin (20 μM) and apyrase (40 U/ml) and then lysed. Complex formation with mt-Hsp70 was analysed by co-immunoprecipitation with an antibody against the mt-Hsp70 protein, Ssc1p (Herrmann *et al.*, 1994).

was also found in a complex with mt-Hsp70, and this level decreased by 55% during the 30 min chase period (Figure 6, lanes 7–9). Thus this complex appeared to be more stable when the pH gradient across the inner membrane was limiting for efficient export.

In summary, ΔpH is required for translocation across the inner membrane of the N-terminal segment preceding the transmembrane segment, whereas matrix ATP is needed for the release of mt-Hsp70 from the Su9 segment of the matured precursor protein.

Discussion

This study provides insights into the sorting pathways of sorting of mitochondrial integral inner membrane proteins. Subunit 9 of the F_1F_0-ATPase of *N.crassa*, a nuclearly encoded protein, can be efficiently imported and correctly sorted in yeast mitochondria, although in yeast the same protein is encoded by a mitochondrial gene. Thus, following import a protein has the ability to embark on the sorting pathway taken by its mitochondrially encoded counterpart (Figure 7).

Despite its hydrophobicity, the first transmembrane domain of Su9 does not act as a stop–transfer signal to arrest the protein in the inner membrane during import. It functions rather as a membrane insertion signal operating from the trans side of inner membrane (Figure 7). In addition to Su9, many other mitochondrial inner membrane proteins contain their N-termini in the intermembrane space. Analysis of the sorting mechanisms of some of these proteins has indicated that their transmembrane domains act also as insertion signals from the trans side of the inner membrane (J.M.Herrmann, H.Fölsch and R.A.Stuart, in preparation). This indicates that the conservative sorting mechanism applies to a number of integral inner membrane proteins. Are all inner membrane proteins sorted in a conservative manner? A number of results would suggest this may not be the case, the best documented protein being cytochrome oxidase subunit Va. This protein, of N_{in}–C_{out} topology, appears not to be sorted via the matrix but rather directly from the level of the inner membrane (Miller and Cumsky, 1993). A decisive step in the sorting of proteins by both mechanisms would

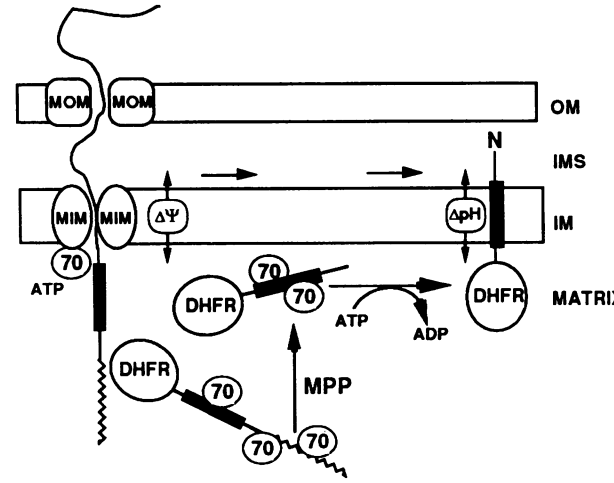

Fig. 7. Model of the import and sorting pathway of preSu9(1–112)–DHFR. PreSu9(1–112)–DHFR is imported into mitochondria in a coordinated fashion across both mitochondrial membranes. Translocation as an extended polypeptide chain, across outer and inner membranes is facilitated by the MOM and MIM translocation machineries, respectively. PreSu9(1–112)–DHFR is initially imported into the matrix in a matrix ATP/mt-Hsp70- and membrane potential-dependent manner. The DHFR domain becomes folded in the matrix. In the absence of MPP activity, unprocessed preSu9(1–112)–DHFR accumulates in the matrix in a translocation-competent manner complexed to mt-Hsp70. Following MPP processing to mSu9(1–112)–DHFR and release from mt-Hsp70, the transmembrane domain inserts into the inner membrane and the N-terminal tail becomes exported into the intermembrane space. This translocation process is supported by a ΔpH gradient across the inner membrane. Abbreviations: MOM, mitochondrial outer membrane translocation machinery; MIM, mitochondrial inner membrane machinery; 70, mt-Hsp70; MPP, mitochondrial processing peptidase; OM, outer membrane, IMS, intermembrane space; IM, inner membrane. The zig-zag corresponds to the 66 amino acid long presequence of Su9 and the filled box corresponds to the transmembrane domain.

come at the level of the inner membrane import machinery. How does this translocation machinery distinguish between those sorting signals which on the one hand could be arrested and sorted laterally, from those on the other hand which must be initially translocated into the matrix? Hydrophobicity is a characteristic motif in both types of sorting signal. A more detailed dissection of these targeting signals is required before conclusions can be drawn about the particular features necessary for specific sorting by one mechanism rather than the other. Interestingly, when translocation across the inner membrane was prevented by the inhibition of mt-Hsp70 action, we observed that preSu9(1–112)–DHFR could become anchored stably at the level of the inner membrane. Stabilization in this manner was sufficient to allow complete MPP processing and translocation across the outer membrane (albeit to a lesser extent than in the presence of functional mt-Hsp70). Thus, impaired mt-Hsp70 function *in vitro* not only leads to incomplete translocation, but also causes efficient localization of a conservatively sorted protein to the intermembrane space. This occurs presumably, however, by an artificial opportunistic lateral sorting pathway.

The import and sorting events of preSu9(1–112)–DHFR are two distinct processes. Following import, the protein can be accumulated in the matrix and then can become sorted to its final topology (N_{out}–C_{in}) across the inner membrane by undergoing an insertion step from the matrix

side (Figure 7). Sorting of cytochrome b_2 and c_1 appears to differ from that of Su9, with respect to the coupling of import and sorting. The precursors of these cytochromes undergo sorting concomitantly with import, as they can be found spanning the sites of import and export looping through the matrix (Gruhler *et al.*, 1995).

The translocation of hydrophilic domains across the mitochondrial inner membrane is strongly influenced by flanking sequences. Retention of the matrix-targeting sequence of Su9 led to a complete block of insertion into the inner membrane. This result is reminiscent of the findings with transmembrane proteins in the bacterial system. There, positively-charged amino acids tend to be more prevalent in the cytoplasmic than in periplasmic regions flanking the transmembrane segments (positive-inside rule) (von Heijne, 1989; Boyd and Beckwith, 1990; Dalbey, 1990). Furthermore, introduction of positive charges in the N-terminal tails of bacterial proteins caused a complete block of translocation (Cao and Dalbey, 1994). Thus, N-terminal translocation occurs in mitochondria in a similar manner as in *Escherichia coli*. This reflects a conservation of sorting principles from prokaryotes to eukaryotic organelles.

Finally, our data show that translocation of the N-terminal tail is supported by a $\Delta\mu H^+$ across the inner membrane (Figure 7). Export was more sensitive to the addition of nigericin than of valinomycin, suggesting it was the ΔpH component which was primarily supporting the export. This observation highlights yet another similarity to the bacterial system, where export of N-terminal tails has been shown to be influenced by the proton motive force (Whitley *et al.*, 1994). In *E.coli* a membrane potential was shown to be necessary to support the translocation of negatively charged N-terminal tails (Andersson and von Heijne, 1994; Cao *et al.*, 1995). Requirement for a ΔpH has also been described for protein translocation across thylakoid membranes (Mould and Robinson, 1991; Cline *et al.*, 1993), emphasizing the conservation of prokaryotic sorting features in eukaryotic organelles.

In summary, our findings suggest that several aspects of protein sorting in eukaryotic organelles have been conserved from their prokaryotic ancestors. In bacteria, short N-terminal tails can be translocated efficiently across the plasma membrane without the aid of leader sequences and in a manner which does not require the Sec machinery (Andersson and von Heijne, 1993; von Heijne, 1994; Whitley *et al.*, 1994). Whether a proteinaceous machinery exists to facilitate the translocation of these N-terminal tails in mitochondria is as yet unclear and will be the focal point of future research.

Materials and methods

Isolation of mitochondria and protein import

Standard procedures for cell growth (*Saccharomyces cerevisiae* wild-type, D273–10B) and mitochondrial isolation were used (Herrmann *et al.*, 1994a). PreSu9(1–112)–DHFR and preSu9(1–69)–DHFR (Ungermann *et al.*, 1994) were synthesized in rabbit reticulocyte lysate (Promega Corp., USA) in the presence of [^{35}S]methionine as previously described (Pelham and Jackson, 1976). Unless otherwise indicated, import was performed as described before in the following buffer: 3% (w/v) 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, 1 mM $MnCl_2$ (buffer A) (Stuart *et al.*, 1994b). Following import samples were treated with trypsin (20 μg/ml) for 15 min on ice.

Hypotonic swelling of mitochondria was performed to determine the extent of export of the N-terminal tail as follows: after trypsin treatment mitochondria were re-isolated (Sigma, rotor 12154, 10 min, 9200 *g*, 2°C) and resuspended in buffer A at a concentration of 25 μg/100 μl containing soybean trypsin inhibitor (0.2 mg/ml) and then were diluted 10-fold in 20 mM HEPES, pH 7.2, in the absence or presence of proteinase K (40 μg/ml), as indicated. 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 proteinase K treatment, as indicated. Samples were kept on ice for 30 min and PMSF (2 mM) was added. Mitoplasts/mitochondria were re-isolated and washed once with SHKCl–PMSF buffer (0.6 M sorbitol, 20 mM HEPES, pH 7.2, 80 mM KCl, 1 mM PMSF) and then lysed directly in SDS-sample buffer. Samples were analysed by SDS–PAGE and immunoblotting to nitrocellulose. The efficiency of swelling was assessed following immunodecoration of the blot with antisera against endogenous cytochrome *c* peroxidase (soluble intermembrane space protein) and Mge1p (matrix located protein).

Accumulation of preSu9(1–112)–DHFR in matrix and chase reaction

The import of preSu9(1–112)–DHFR was performed for 30 min at 12°C in the following buffer: 3 % (w/v) BSA, 50 mM HEPES, pH 7.2, 0.5 M sorbitol, 80 mM KCl, 2 mM K-phosphate, 10 mM EDTA, 2 mM *o*-phe (buffer B). Samples were placed on ice and treated with trypsin (20 μg/ml) for 15 min. Mitochondria were re-isolated by centrifugation (Sigma, rotor 12154, 10 min, 9200 *g*, 2°C) and were resuspended in fresh import buffer containing either 10 mM EDTA/2 mM *o*-phe (buffer B) or in buffer A. Samples were chased for indicated times at 12°C. Osmotic swelling in presence of proteinase K was performed to determine the efficiency of export of N-terminal tail, as described above.

Antibody production

The synthetic peptide SSEIAQAMVEVSKC corresponding to the N-terminus of Su9 (*N.crassa*) was coupled to keyhole limpet haemocyanin with maleimide carrier protein (Imject, Pierce) and was used for the generation of antibodies in rabbits

Miscellaneous

The following procedures were performed according to published methods, protein concentration determination (Bradford, 1976); mt-Hsp70 co-immunoprecipitation analysis (Herrmann *et al.*, 1994b); SDS–PAGE (Laemmli, 1970).

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