Import of rat mitochondrial citrate carrier (CIC) at increasing salt concentrations promotes presequence binding to import receptor Tom20 and inhibits membrane translocation

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Summary

Mitochondria contain a family of related carrier proteins that mediate transport of metabolites across the mitochondrial inner membrane. All members of this family are synthesized in the cytosol. We characterized the interactions of newly synthesized rat citrate carrier (CIC) precursor protein (pCIC) with the components of the mitochondrial protein import machinery. pCIC contains both a positively charged presequence of 13 amino acids and internal targeting sequences. We found that the pCIC presequence does not interfere with the import pathway and merely acts as an internal chaperone in the cytosol. Under conditions of increased ionic strength, the pCIC presequence binds to the import receptor Tom20 and accumulates at the mitochondrial surface, thereby delaying pCIC translocation across the mitochondrial outer membrane. Similarly, the presequence of the bovine phosphate carrier (PiC) precursor protein (pPiC) is arrested at the mitochondrial surface when salt concentrations are elevated. We conclude that presequences can only act as mediators of mitochondrial protein import if they allow rapid release from import receptor sites. Release from receptors sites may be rate-limiting in translocation.

Key words: Citrate carrier, Mitochondria, Membrane translocation

Introduction

Protein targeting to mitochondria is mediated by several different signal sequences (Neupert, 1997; Rapaport, 2003). Most mitochondrial proteins originate from precursor proteins that are synthesized in the cytosol and expose a cleavable presequence at the N-terminus (Schatz, 1987; Roise and Schatz, 1988; Hartl et al., 1989). Presequences of this type interact with the mitochondrial outer-membrane protein Tom20 (translocase of the mitochondrial outer membrane, component of 20 kDa). Some other mitochondrial proteins show binding to a different import receptor, Tom70, using specific internal targeting sequences. Representatives of this group of pre-proteins are members of the inner membrane metabolite carrier family. The human genome probably encodes 48 carrier proteins, the yeast genome revealed 35 homologs (Palmieri et al., 2000; Kunji, 2004). It is clear that interactions of pre-proteins with receptor sites at the mitochondrial surface play an important role in mediating both targeting and efficient translocation across the mitochondrial membranes, whereas surprisingly little is known about the mechanisms and forces involved in this process.

In most studies on mitochondrial import of carrier proteins, the ADP/ATP carrier (AAC) of the yeast *Saccharomyces cerevisiae* was used as a model protein (Pfanner and Neupert, 1987; Koehler et al., 1999; Bauer et al., 2000; Zara et al., 2003a; Rehling et al., 2003; Rehling et al., 2004). Detailed investigations led to the definition of five distinct stages of the AAC import pathway. Like all other mitochondrial carrier proteins, the AAC is synthesized in the cytosol (stage I). Mediated by three internal sequences, the AAC binds to the import receptor Tom70 at the mitochondrial outer surface (stage II). Tom70 facilitates binding to the TOM complex that mediates protein translocation across the outer membrane. The protein-conducting channel is mainly formed by the subunit Tom40. At the inner exit of the channel, the AAC binds to a soluble complex of the intermembrane space proteins Tim9 and Tim10 (translocase of the mitochondrial inner membrane, components 9 and 10). If the mitochondrial membrane potential is dissipated, the AAC accumulates at this site, defining stage III of the import pathway. Under normal conditions, the membrane potential initiates insertion of the AAC into the TIM22 complex of the inner membrane (stage IV) and formation of functional AAC homo-dimers in the lipid bilayer (stage V).

Other proteins of the mitochondrial inner membrane show significant differences from this scheme (Neupert, 1997;

Rehling et al., 2003). They are synthesized as precursor proteins containing an N-terminal presequence and target Tom20 as major import receptor. Insertion into the mitochondrial inner membrane is mediated by the TIM23 complex. Tim9/Tim10 and the TIM22 complex are not involved. Particularly interesting is the fate of the inner-membrane-protein Oxa1p (Herrmann et al., 1997; Kuhn et al., 2003). Similar to the carrier proteins, Oxa1p is a hydrophobic inner-membrane-protein containing several membrane-spanning segments. However, in contrast to the AAC, Oxa1p contains a presequence that directs the pre-protein to the TIM23 complex. Insertion into the inner membrane is only possible in the presence of intact Tim44 and mtHsp70.

The mechanisms of mitochondrial protein import appear to be highly conserved in evolution (Rassow and Pfanner, 2000; Koehler, 2004). It was therefore a surprise to see that, in mammals and in plants some carrier proteins are synthesized as pre-proteins containing a cleavable N-terminal presequence (Palmieri et al., 1996; Zara et al., 2003a; Murcha et al., 2004). The presequences contain several positively charged amino acid residues that are typical for mitochondrial targeting sequences. Previous studies on bovine phosphate carrier (PiC) and rat citrate carrier (CIC) showed that the mature proteins that lack the presequence (mPiC and mCIC, respectively) contained sufficient targeting information to allow efficient import into mitochondria (Zara et al., 1992; Zara et al., 2003b). Since data on the interactions of carrier presequences with the mitochondrial TOM and TIM complexes are lacking, it is still unclear whether these presequences direct their cargo on a Tom20-TIM23-Tim44/mtHsp70 pathway (similar to Oxa1p) or whether the carrier proteins are kept in the track of the Tom70-Tim9/10-TIM22 pathway, following the conventional stages I-V.

In this study we investigated the biogenesis of the CIC precursor protein (pCIC, tricarboxylate carrier) (Kaplan et al., 1993) and studied its interactions with distinct components of the mitochondrial protein import machinery. The CIC presequence comprises 13 residues including two arginines (MAAPRAPRALTAA). We compared the import pathway of the CIC precursor protein (pCIC) with that of the mature citrate carrier (mCIC) protein. The results show that under normal conditions the CIC presequence does not interfere with the CIC import pathway in any stage. The situation changes at increasing salt concentrations. Under conditions of high ionic strength, the pCIC presequence binds to the import receptor Tom20. However, this interaction does not entail an increase in the rate of import but an inhibition of translocation. Similar observations were made with the bovine PiC precursor protein (pPiC). We conclude that mitochondrial presequences are not optimised for strong binding but for an equilibrium that allows both specific binding and rapid release.

Materials and Methods

Pre-proteins and mitochondria

Pre-proteins were synthesized in rabbit reticulocyte lysate (TNT™ coupled reticulocyte lysate system, Promega) in the presence of ^{35}S-methionine. Plasmids encoding the pre-proteins were constructed using vectors of the pGEM series (Promega). The cloning procedures are described elsewhere [rat CIC (Zara et al., 2003b), bovine PiC (Zara et al., 1991; Zara et al., 1992), AAC of *Neurospora crassa* (Pfaller et al., 1988) and Su9-DHFR containing the N-terminal 66 amino acid residues of subunit 9 of *Neurospora crassa* mitochondrial ATP synthase fused to the dihydrofolate reductase of the mouse (Pfanner et al., 1987)].

For isolation of mitochondria, yeast cells were grown in YPG medium [1% (w/v) yeast extract, 2% (w/v) bacto-peptone pH 5.0, containing 3% (v/v) glycerol]. Mitochondria were isolated from rat liver and *S. cerevisiae* by standard procedures (Rassow, 1999) and stored in SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS-KOH, pH 7.2). For import experiments involving different types of mitochondria, mutant and wild-type mitochondria were isolated in parallel at the same day, using genetically equivalent strains and identical growth conditions. The yeast strains used in this study are listed in Table 1. Import experiments that only required a wild-type strain were performed using mitochondria of the strain PK82 (Gambill et al., 1993).

To open the mitochondrial outer membrane, isolated mitochondria (30 μg of protein) were suspended in 50 μl 10 mM HEPES-KOH pH 7.2. The sample was incubated at 0°C for 20 minutes. The mitoplasts were isolated by centrifuging the samples for 30 minutes at 64,000 ***g*** (2°C).

Import of pre-proteins into isolated mitochondria

Import of radiolabeled pre-proteins into mitochondria was essentially performed as described previously (Rassow, 1999; Zara et al., 1992; Zara et al., 2003b). Mitochondria from temperature-sensitive yeast strains (*tim44-8* and *ssc1-3*) were incubated for 15 minutes at 37°C in import buffer to induce the phenotype.

To block Tom20 and Tom70 of rat liver mitochondria, the isolated organelles (100 μg of protein) were incubated with polyclonal rabbit antibodies at concentrations as indicated, in a volume of 50 μl for 30 minutes at 0°C. The mitochondria were again isolated by centrifugation and then used in subsequent import assays.

Pretreatment of mitochondria with trypsin was carried out at 0°C for 15 minutes. Mitochondria (20 μg of protein) were suspended in 200 μl SEM buffer and incubated with 20 μg trypsin (bovine pancreas trypsin, type XIII, Sigma T8642). The reaction was stopped by addition of 600 μg trypsin inhibitor (Soybean trypsin inhibitor, Type I-S, Sigma T9003). In subsequent steps, 0.5 mg/ml trypsin inhibitor were included to block the activity of residual protease.

For import of pre-proteins, suspensions containing mitochondria from rat liver (75 μg of protein) or from *S. cerevisiae* (30 μg of protein) and reticulocyte lysate were diluted with BSA-buffer (3% (w/v) BSA, 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 10 mM MOPS-KOH pH 7.2) to a final volume of 100 μl. ATP (2 mM) was added from a 100-fold concentrated stock solution. Sodium succinate (10 mM) or NADH (2 mM) were added to rat liver and *S. cerevisiae*

Table 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Reference
MM208 (*tom70Δ*)	*ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801 tom70::HIS3*	Moczko et al. (1994)
MM112-C (*tom20Δ*)	*ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801 tom20::URA3*	Moczko et al. (1994)
KD56 (*tom5Δ*)	*ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801 tom5::HIS3*	Dietmeier et al. (1997)
OL201 (*tom22Δ*)	*his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 tom22::HIS3 rho*$^-$	van Wilpe et al. (1999)
OL200-AH49 (*tom22-2*)	*his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 tom22::HIS3+pRS414(TRP1)-tom22-2*	Moczko et al. (1997)
PK82	*his4-713 lys2 ura3-52Δ trp1 leu2-3, 112*	Gambill et al. (1993)
PK83 (*ssc1-3*)	*ade2-101 lys2 ura3-52Δ trp1 leu2-3, 112 ssc1-3(LEU2)*	Gambill et al. (1993)

mitochondria, respectively. Where indicated with '–Δψ', 1 μM valinomycin and 20 μM oligomycin were added to isolated mitochondria to dissipate the membrane potential. All import reactions were carried out at 25°C. For protease treatment, the samples were cooled to 0°C and incubated with proteinase K for 20 minutes at 0°C. The samples were subsequently incubated with 3 mM phenylmethylsulfonylfluoride (PMSF) for 5 minutes at 0°C. The mitochondria were again isolated by centrifugation and analysed by SDS-PAGE and fluorography.

Results

CIC targeting to import receptor Tom70 is independent of the presequence

We investigated the role of the pCIC presequence by comparing mitochondrial import of the CIC precursor protein (pCIC) with that of the mature CIC (mCIC), which lacks the presequence. Both proteins were synthesized in reticulocyte lysate in the presence of ^{35}S-labeled methionine. Samples of freshly isolated rat liver mitochondria were preincubated in the presence of increasing concentrations of polyclonal antibodies directed against rat Tom70 or Tom20 (Fig. 1A) (Suzuki et al., 2002). The mitochondria were then incubated at 25°C with reticulocyte lysate containing either pCIC or mCIC. The incubation was stopped after 20 minutes, the samples were cooled to 0°C and non-imported CIC was removed by treatment with proteinase K. The mitochondria were then isolated by centrifugation and analyzed by SDS-PAGE. The relative amount of imported CIC was determined using a phosphoimager. As observed in previous studies on import of other carrier proteins into mitochondria of *Neurospora crassa*, yeast and mammals (Söllner et al., 1990; Steger et al., 1990; Suzuki et al., 2002), uptake of the CIC proteins was strongly inhibited by the antibodies directed against Tom70. Inhibition by the antibodies directed against Tom20 was less efficient but still significant, similar to data previously published (Steger et al., 1990; Suzuki et al., 2002). Remarkably, the effects on pCIC and mCIC were almost identical (Fig. 1A, left panel vs right panel). The presequence of pCIC had no obvious influence on the recognition of the TOM complex. It is particularly noteworthy that the CIC presequence did not shift the targeting from Tom70 to Tom20.

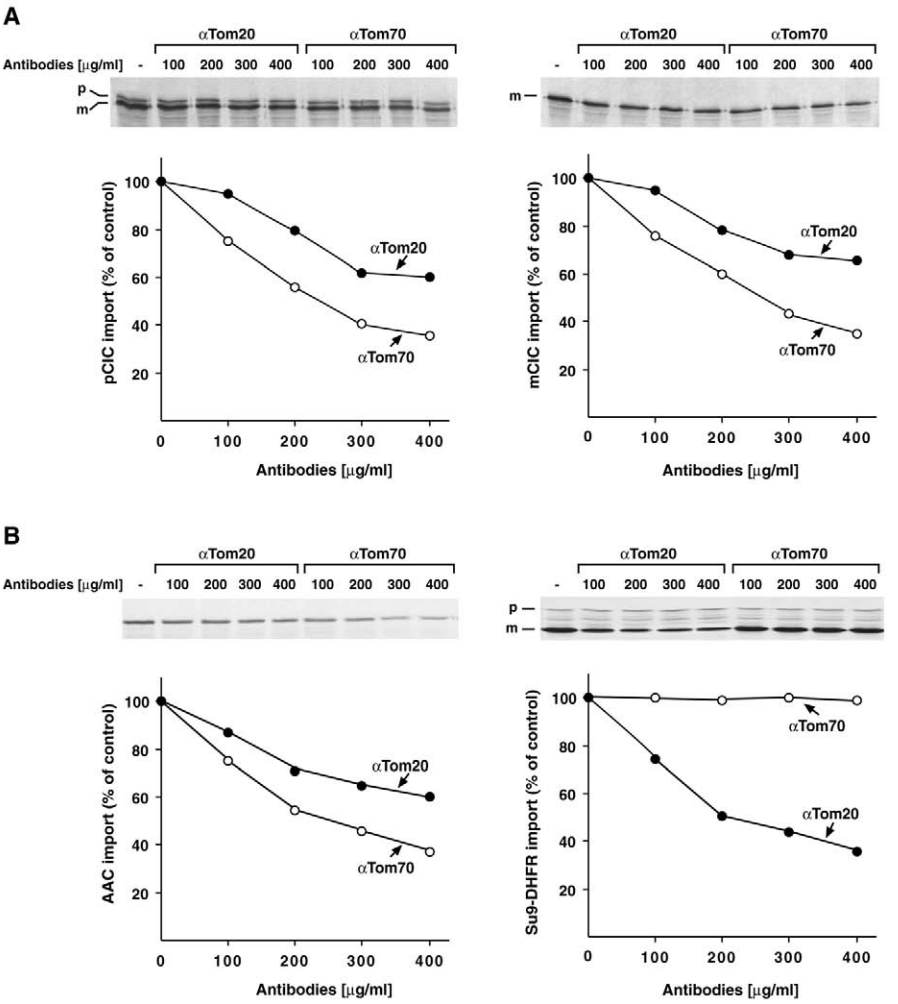

Fig. 1. Import of CIC into isolated rat liver mitochondria. (A) Isolated rat liver mitochondria were preincubated with antibodies directed against Tom20 or Tom70, respectively, at increasing concentrations. Reticulocyte lysate containing the ^{35}S-labeled pCIC or mCIC was added and the samples were incubated for 20 minutes at 25°C. The mitochondria were then cooled-down to 0°C and treated with 50 μg/ml proteinase K to remove non-imported protein. Proteolysis was stopped by addition of PMSF, the mitochondria were isolated again by centrifugation, and imported proteins were analyzed by SDS-PAGE and autoradiography. The relative amounts of ^{35}S-labeled protein were determined using a phosphoimager. The amounts of imported protein in the absence of antibodies were set to 100% (control). (B) Import of AAC and Su9-DHFR (a hybrid protein containing the presequence of subunit 9 of the mitochondrial ATP synthase coupled to dihydrofolate reductase), following the same protocol as in the experiments of A. p, precursor protein; m, mature protein.

In parallel experiments, we imported AAC into mitochondria of the same preparation (Fig. 1B, left panel). The pattern of inhibition closely resembled the pattern obtained with the CIC. By contrast, import of Su9-DHFR, an artificial model protein containing an N-terminal presequence (Pfanner et al., 1987; Rassow, 1999), was only inhibited by antibodies directed against Tom20 (Fig. 1B, right panel). The data indicate that import of both pCIC and mCIC depends on the same receptor structures as import of other members of the carrier family.

To obtain independent evidence, we used mutants of the yeast *Saccharomyces cerevisiae* as an alternative test system (Fig. 2). pCIC and mCIC were again synthesized in reticulocyte lysate and incubated with isolated mitochondria. The organelles were isolated from deletion strains lacking the genes encoding Tom20 or Tom70, corresponding wild-type

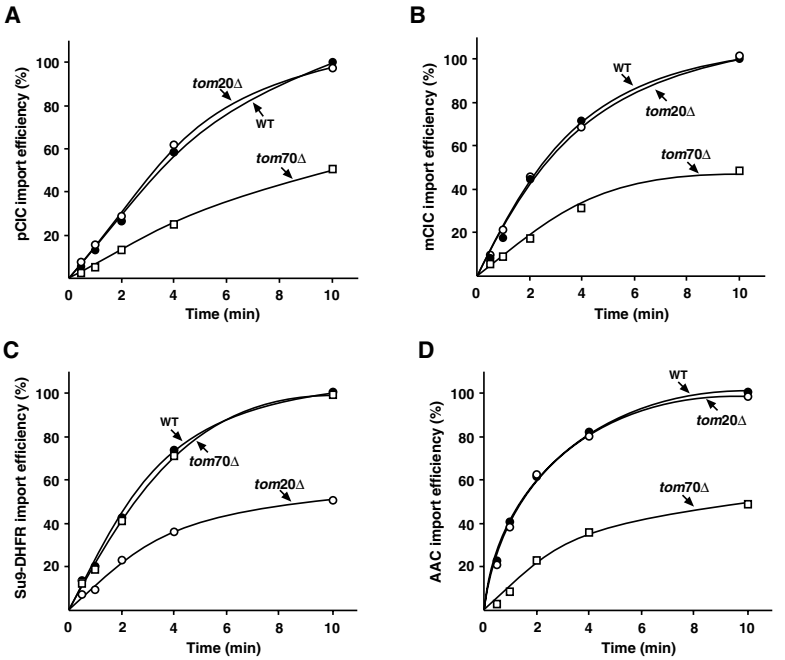

Fig. 2. Import of CIC into isolated yeast mitochondria. (A) Mitochondria were isolated from yeast strains lacking the import receptor Tom70 (*tom70Δ*) or Tom20 (*tom20Δ*). Wild-type mitochondria (WT) were isolated in parallel. ^{35}S-labeled pCIC was synthesized in reticulocyte lysate and incubated with the isolated mitochondria at 25°C. Aliquots were removed at different time points as indicated and cooled-down to 0°C. The mitochondria were incubated with 250 μg/ml proteinase K for 20 minutes and isolated again by centrifugation. Imported proteins were analyzed by SDS-PAGE and fluorography. The relative amount of imported pCIC was determined using a phosphoimager. The highest value was set to 100% (control). Within import-times of 10 minutes, up to 11% of added pCIC were imported. (B) Import of mCIC, following the same protocol as described in A. (C) Import of Su9-DHFR. (D) Import of AAC.

strains were used for comparison. In agreement with the data obtained with rat liver mitochondria, import of pCIC and mCIC was significantly reduced in mitochondria lacking Tom70 (Fig. 2A,B). Import into mitochondria lacking Tom20 showed an efficiency that was similar to import efficiency into wild-type mitochondria. Su9-DHFR was again used as a reference protein because it contains a classic N-terminal presequence targeting Tom20 (Fig. 2C). AAC was imported as an alternative carrier protein (Fig. 2D). The results clearly demonstrate that the presequence of pCIC does not modify the involvement of the two import receptors Tom70 and Tom20 in targeting of the mature CIC protein. We conclude that mitochondrial targeting of pCIC is essentially mediated by Tom70, thus following the same pattern as the AAC and other members of the carrier family. The CIC presequence does not interfere with this process.

CIC translocation through the outer membrane TOM channel is independent of the presequence

Within the TOM complex, the import receptors Tom20 and Tom70 are functionally linked to the general import pore (GIP) by Tom5. Mitochondria lacking Tom5 show a delay in the uptake of nearly all pre-proteins that traverse the mitochondrial outer membrane (Dietmeier et al., 1997). We tested

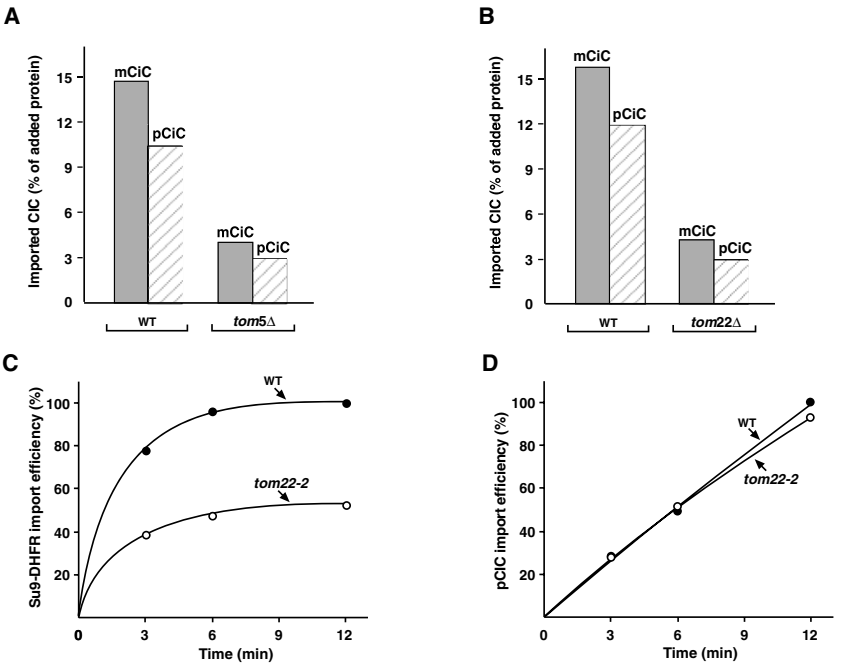

Fig. 3. Import of CIC into mitochondria that lack components of the TOM complex. (A) ^{35}S-labeled pCIC and mCIC were synthesized in reticulocyte lysate and incubated for 10 minutes at 25°C with isolated yeast mitochondria lacking Tom5. Mitochondria of the corresponding wild type were used in parallel assays. The import reaction was stopped by treatment with 250 μg/ml proteinase K at 0°C. The mitochondria were isolated again and the mitochondrial proteins were separated by SDS-PAGE. The relative amounts of imported CIC were determined using a phosphoimager. (B) Import of ^{35}S-labeled pCIC and mCIC into mitochondria that lack Tom22, following the same protocol as described in (A). (C) Import of ^{35}S-labeled Su9-DHFR into isolated mitochondria of the yeast strain *tom22-2*. (D) Import of pCIC into *tom22-2* mitochondria.

mitochondria from a *tom5Δ* deletion strain and mitochondria from the corresponding wild-type strain for import of the CIC (Fig. 3A). The results were very similar with pCIC and mCIC. In the *tom5Δ* mitochondria the import of both proteins was inhibited to the same extent, suggesting that the CIC presequence is not involved in the Tom5-dependent transfer of pCIC from the receptor sites to the GIP.

Tom22 is known to be a central organizer of the TOM complex. Depletion of Tom22 causes complete disintegration of the complex (van Wilpe et al., 1999). To test for the relevance of the TOM complex in the import of CIC, we isolated mitochondria from a *tom22Δ* yeast strain. In the in vitro import assay pCIC and mCIC were again similarly affected (Fig. 3B), indicating that the requirements for pCIC and mCIC in the interactions with the TOM complex are identical. The transfer of pCIC across the outer membrane appears to follow the same pathway as described for other carrier proteins. The N-terminal presequence does not modify the interactions with the import receptors or with the general insertion pore.

The import route of pCIC separates from the pathway of other presequence-carrying pre-proteins at the *trans* side of the TOM complex

The general import pore of the TOM complex is used by many different pre-proteins. Their import pathways separate at the inner exit of the pore. The AAC and other carrier proteins bind to Tim9/Tim10 for transfer to the TIM22 complex of the inner membrane. Oxa1p and similar proteins carrying a cleavable presequence are handed over to the TIM23 complex. In yeast, the exit side of the TOM complex is marked by the intermembrane space domain of Tom22 that exposes several negatively charged amino acids. The charges are thought to attract positively charged presequences and to contribute to the mechanism of pre-protein translocation (Bolliger et al., 1995; Moczko et al., 1997; Schatz, 1997). Although carrier proteins, such as the AAC, expose positive charges at internal residues, these residues do not bind to the intermembrane space domain of Tom22. It was therefore suggested that the *trans* side of Tom22 interacts specifically with N-terminal presequences (Kübrich et al., 1998). In agreement with this, we found that import of Su9-DHFR was delayed in mitochondria of the mutant *tom22-2* (Fig. 3C). In this mutant, the gene encoding Tom22 is not completely deleted, only the C-terminal part containing the negatively charged residues is missing (Moczko et al., 1997). In contrast to Su9-DHFR, the import kinetics of pCIC were similar in *tom22-2* and in wild-type mitochondria (Fig. 3D). Identical import into wild-type and *tom22-2* mutant mitochondria were also observed with mCIC and with the AAC (not shown). In contrast to the presequence of Su9-DHFR, the N-terminal presequence of pCIC apparently does not recognize the *trans* side of Tom22.

We next asked whether the presequence of pCIC is capable of interacting with the TIM23 complex that mediates the insertion of presequences into the mitochondrial inner membrane (Rehling et al., 2003). Several precursor proteins directly insert into the inner membrane if the outer membrane is removed (Hwang et al., 1989). The process is initiated by direct interactions between their presequences and the N-terminal domain of Tim23 (Bauer et al., 1996). By contrast, import of carrier proteins that lack a presequence strictly depends on an intact mitochondrial outer membrane and the content of the intermembrane space. Direct binding of substrate proteins to the TIM22 complex is not possible (Kübrich et al., 1998; Murphy et al., 2001; Curran et al., 2002a; Curran et al. 2002b). By swelling mitochondria in diluted

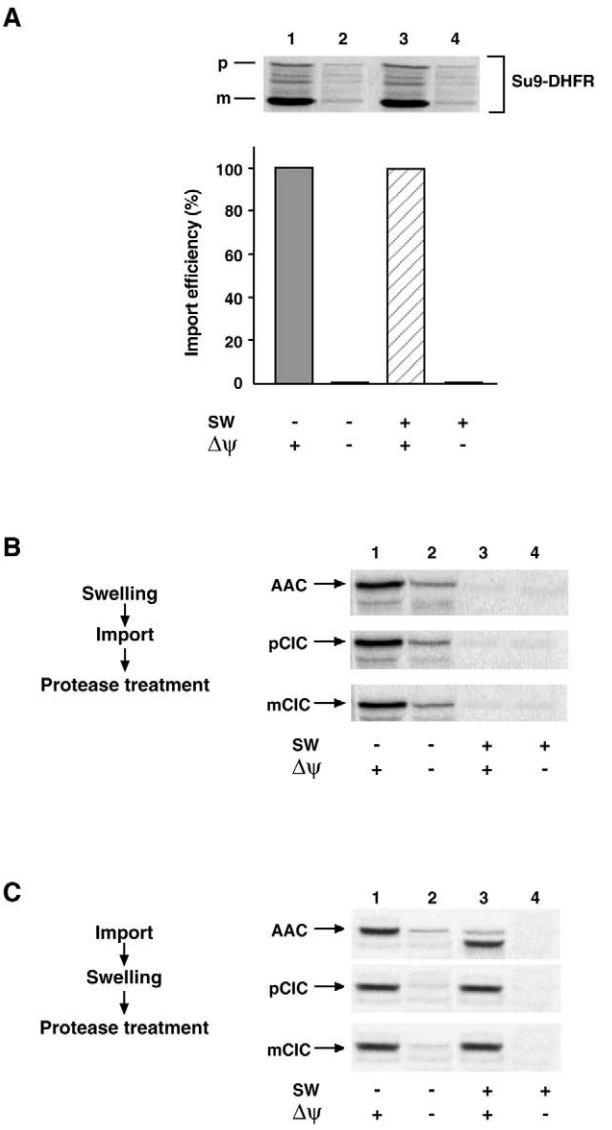

Fig. 4. Import of pre-proteins into mitoplasts. (A) Mitoplasts were prepared by osmotic shock (swelling, SW) of yeast mitochondria. Reticulocyte lysate containing ^{35}S-labeled Su9-DHFR was incubated for 20 minutes at 25°C with mitochondria (lanes 1 and 2) or mitoplasts (lanes 3 and 4). Valinomycin and oligomycin were added to samples 2 and 4 to dissipate the membrane potential (–Δψ). To degrade non-imported protein, 50 μg/ml proteinase K were subsequently added to all samples. The mitochondria were isolated again and analyzed by SDS-PAGE and fluorography. (B) Import of AAC, pCIC and mCIC. The experiment was performed as described in A. (C) Import of AAC, pCIC, and mCIC into intact mitochondria. The ^{35}S-labeled proteins were imported into intact yeast mitochondria for 20 minutes at 25°C. The mitochondria of samples 3 and 4 were subsequently subjected to osmotic shock (+ SW). Following treatment with proteinase K, mitochondria and mitoplasts were isolated again by centrifugation.

buffer, we opened the intermembrane space and tested for the import of several proteins (Fig. 4). Using Su9-DHFR and AAC as established model proteins (Kübrich et al., 1998), we confirmed that, after opening the intermembrane space, import of Su9-DHFR was still possible (Fig. 4A, lane 1 vs lane 3), whereas import of the AAC was completely blocked (Fig. 4B, upper panel, lane 1 vs lane 3). In parallel experiments, we tested pCIC and mCIC under the same conditions. The results clearly demonstrate that after the rupture of the outer membrane, neither pCIC nor mCIC show any residual import (Fig. 4B, lower panel). In control samples, we first imported the carrier proteins into intact mitochondria and then tested for the accessibility of the imported proteins upon opening of the outer membrane (Fig. 4C). In these samples both the AAC and the CIC were efficiently imported and partially (AAC) (Rassow and Pfanner, 1991; Kübrich et al., 1998) or completely (CIC) protected against the protease by insertion into the membrane (Fig. 4C, lane 3). The CIC seems to follow the same pathway into the inner membrane as the AAC. It is unlikely that the presequence of pCIC targets Tim23.

Insertion of pCIC into the mitochondrial inner membrane is independent of Tim44 and matrix heat shock protein mtHsp70

Precursor proteins carrying an N-terminal presequence usually target the TIM23 complex and come into contact with mtHsp70, the major heat shock protein of 70 kDa in the mitochondrial matrix. The interaction with mtHsp70 is mainly mediated by Tim44, a hydrophilic protein that is associated with the inner side of the TIM23 complex (Neupert, 1997; Rehling et al., 2003). To test for a role of Tim44 in the import of pCIC, we isolated mitochondria from the temperature-sensitive yeast mutant *tim44-8* (Bömer et al., 1998). Isolated mitochondria were preincubated for 15 minutes at 37°C to inactivate the mutant Tim44, wild-type mitochondria were treated in the same way. With Su9-DHFR, the *tim44-8* mitochondria showed a clear phenotype: the import of Su9-DHFR was significantly reduced. By contrast, the import rates of pCIC were essentially the same in mutant and wild-type mitochondria (Fig. 5A,B). The import of AAC, performed as a control, was also unaffected in the *tim44-8* mitochondria (Fig. 5C). However, these results do not necessarily exclude a participation of mtHsp70 in the insertion of pCIC into the inner membrane (Bömer et al., 1998; Geissler et al., 2000; Reif et al., 2001).

We therefore used mitochondria from a mutant of mtHsp70 (Fig. 5D-F) in additional experiments. The mitochondria were isolated from the temperature-sensitive yeast strain *ssc1-3*, which was previously described as having a strong phenotype in the import of all pre-proteins that are targeted by a cleavable N-terminal presequence (Gambill et al., 1993; Voos et al., 1993). In our assays, the *ssc1-3* mitochondria showed a strongly reduced import of Su9-DHFR (Fig. 5E), but normal

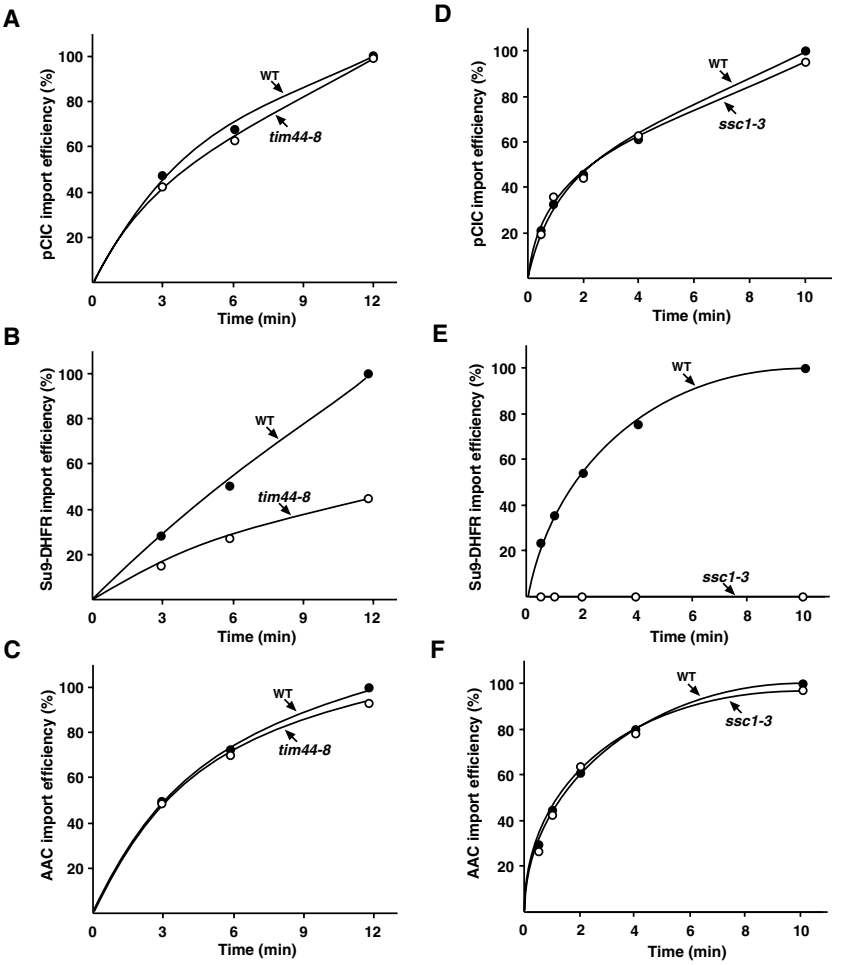

Fig. 5. Participation of Tim44 and mitochondrial Hsp70 (mtHsp70) in import of pre-proteins. (A) Mitochondria were isolated from the temperature-sensitive yeast strain *tim44-8*, wild-type mitochondria (WT) were isolated in parallel under the same conditions. To induce the phenotype, the isolated mitochondria were incubated for 15 minutes at 37°C. The mitochondria were then incubated with reticulocyte lysate containing ^{35}S-labeled pCIC at 25°C. Samples were taken after different time points as indicated, and treated with 250 μg/ml proteinase K for 20 min at 0°C. The proteins were subjected to SDS-PAGE and autoradiography. The relative amounts of imported pCIC were determined using a phosphoimager. The highest value was set to 100% (control). (B) Import of Su9-DHFR. The experiment followed the same protocol as described in A, using mitochondria of the same preparation. (C) Import of AAC. (D) Import of pCIC into mitochondria of the temperature-sensitive yeast strain *ssc1-3*. The gene *SSC1* encodes the major 70 kDa heat shock protein of the mitochondrial matrix. (E) Import of Su9-DHFR into mitochondria from the same preparation as in experiment D. (F) Import of AAC.

import of pCIC (Fig. 5D) and AAC (Fig. 5F). The combined results of Figs 4 and 5 thus indicate that the pCIC presequence does not address either the TIM23 complex or the associated mtHsp70. Similar to other carrier proteins, the biogenesis of pCIC appears to be independent of the matrix mtHsp70 system.

pCIC presequence binding to Tom20 at increasing salt concentrations inhibits translocation across the mitochondrial outer membrane

Import rates of pre-proteins that target different import receptor sites show remarkable differences in their sensitivities to increasing ionic strength (Pfaller et al., 1989; Haucke et al., 1995). In vitro, import is commonly carried out using buffer systems that contain 80 mM KCl. We now imported AAC and Su9-DHFR into yeast mitochondria at concentrations of 80-480 mM KCl. Import was stopped after 10 minutes, samples were treated with proteinase K and we determined the relative amount of imported protein (Fig. 6A). Whereas the import rate of AAC was similar in all conditions, increasing concentrations of KCl strongly inhibited the import of Su9-DHFR. We then repeated the experiment using pCIC and mCIC as reference proteins (Fig. 6B). mCIC resembled the AAC and showed similar import rates at all salt concentrations. The import of pCIC, however, was inhibited by increasing KCl concentrations. This was the first experiment that showed a significant difference between pCIC and mCIC.

High salt concentrations appeared to prevent or to promote interactions of the pCIC presequence with a component of the mitochondrial import machinery. To identify the presequence interaction site, we imported pCIC and mCIC into mitochondria lacking either the import receptor Tom70 or Tom20 (Fig. 6C,D). Import into mitochondria that lack Tom70 (*tom70Δ*) showed the same pattern as import into wild-type mitochondria. However, import of pCIC into mitochondria lacking Tom20 (*tom20Δ*) was no longer inhibited by KCl (Fig. 6C). Import of mCIC was again independent of the KCl concentration (Fig. 6D). The pCIC presequence appears to bind to Tom20 when hydrophobic interactions are facilitated. This conclusion is in agreement with previous studies that showed that N-terminal presequences of matrix-targeted mitochondrial precursor proteins bind to Tom20 by hydrophobic interactions (Brix et al., 1997; Abe et al., 2000). Binding of the pCIC presequence to Tom20 seems to cause a delay in the translocation of the protein across the mitochondrial outer membrane.

To exclude indirect effects of the *TOM20* deletion, we investigated what consequences of a pretreatment with

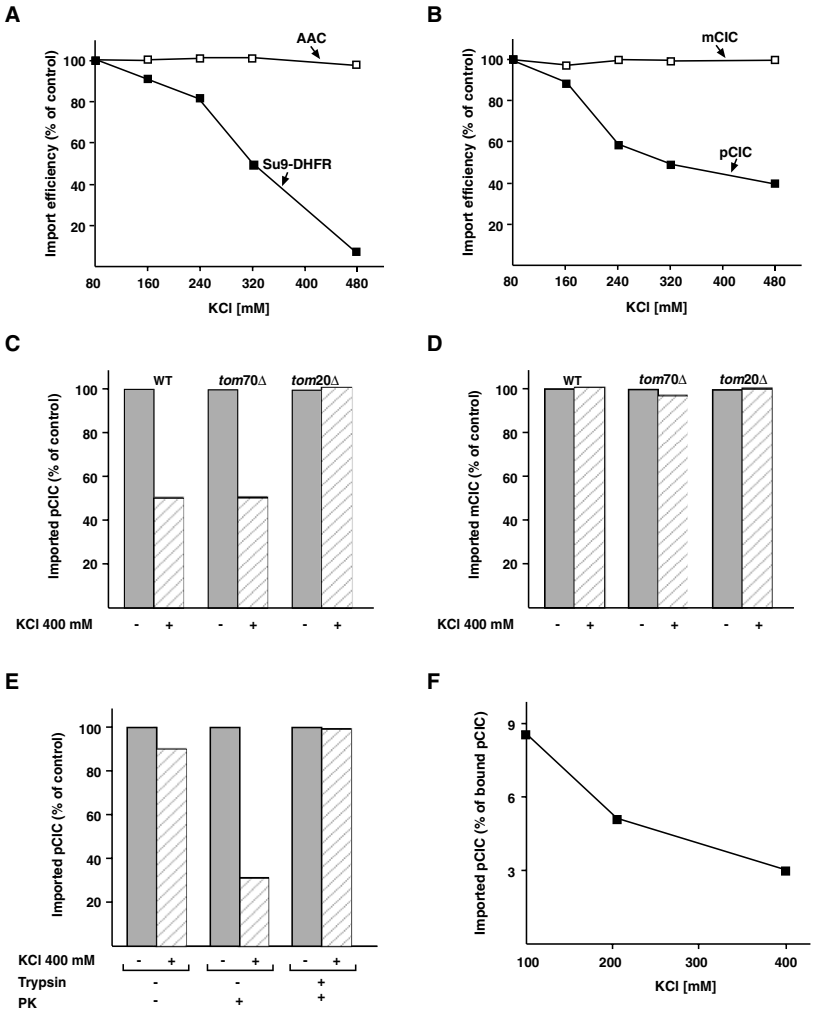

Fig. 6. Inhibition of protein import by increasing concentrations of KCl. (A) AAC and Su9-DHFR were synthesized in reticulocyte lysate in the presence of ^{35}S-labeled methionine. The reticulocyte lysates were diluted with BSA-buffer pH 7.2, containing different concentrations of KCl as indicated. Wild-type yeast mitochondria were added and the mixtures were incubated for 10 minutes at 25°C. The samples were then cooled-down to 0°C and proteinase K was added to digest non-imported protein. The mitochondria were isolated again by centrifugation and analyzed by SDS-PAGE and autoradiography. The relative amounts of imported protein were determined using a phosphoimager. The amounts of AAC or Su9-DHFR that had been imported in the presence of 80 mM KCl were set to 100% (control). (B) Import of pCIC and mCIC, following the same procedure as in A. (C) Import of pCIC into wild-type mitochondria (WT), and into mitochondria lacking the import receptor Tom70 (*tom70Δ*) or Tom20 (*tom20Δ*), respectively. The import experiment essentially followed the scheme described in A, except that the samples contained either 80 mM KCl (–) or 400 mM KCl (+). (D) Import of mCIC, following the same protocol as described in C. (E) Import of pCIC into wild-type mitochondria. The mitochondria were pretreated with 100 μg/ml trypsin for 15 minutes at 0°C (+) or left without trypsin (–). The mitochondria were incubated with pCIC for 10 minutes at 25°C. The samples were subsequently treated with 250 μg/ml proteinase K (+ PK) or left without protease (– PK). The amounts of imported pCIC were determined as described in (A). (F) Relative amounts of imported pCIC vs bound pCIC after import in the presence of different KCl concentrations. The experiment was carried out as described in B, using parallel samples +/– treatment with proteinase K.

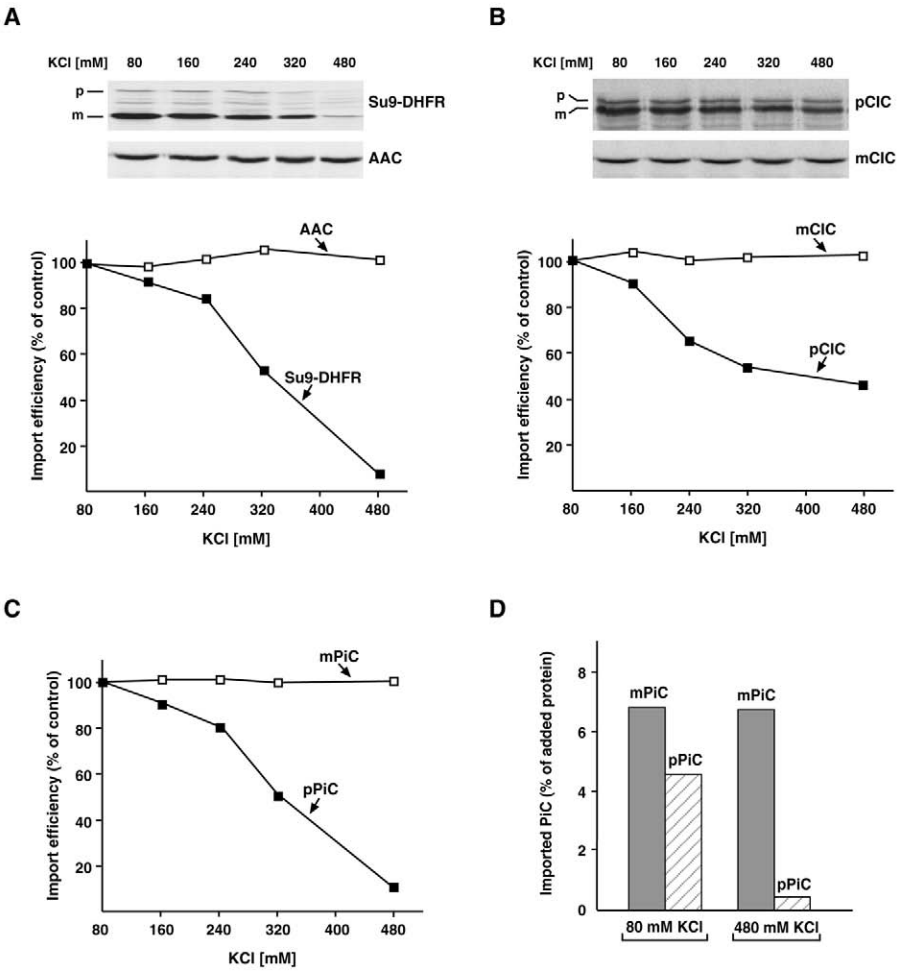

Fig. 7. Inhibition of protein import into both rat liver and yeast mitochondria by high ionic strength. (A) AAC and Su9-DHFR were synthesized in reticulocyte lysate in the presence of ^{35}S-methionine. Isolated rat liver mitochondria were added in the presence of increasing concentrations of KCl as indicated. The samples were incubated at 25°C for 20 minutes, cooled-down to 0°C and treated with proteinase K. The mitochondria were isolated again and analyzed by SDS-PAGE and fluorography. The relative amounts of imported protein were calculated, the values determined for the 80 mM KCl sample were set to 100% (control). (B) Import of pCIC and mCIC, following the same procedure as in A. (C) Precursor (pPiC) and mature phosphate carrier lacking the presequence (mPiC) were synthesized in reticulocyte lysate in the presence of ^{35}S-methionine. Isolated yeast mitochondria (wild-type) were added in the presence of increasing concentrations of KCl as indicated. The samples were then treated as described in (A). (D) pPiC and mPiC were imported into isolated yeast mitochondria as described in C. Standard samples of the reticulocyte lysate were included in the SDS-PAGE to determine the ratio of imported PiC vs the total amount of PiC added to each sample.

protease would have on wild-type mitochondria (Fig. 6E). In the absence or presence of KCl (400 mM), the percentage of pCIC bound to wild-type mitochondria was similar (Fig. 6E, two left columns). Subsequent treatment of the mitochondria with proteinase K (PK) confirmed that KCl inhibited the translocation of pCIC across the outer membrane (Fig. 6E, two middle columns). However, when mitochondria had been pretreated with 100 μg/ml trypsin, the KCl-dependent inhibition of import was lost (Fig. 6E, two right columns). We determined the fraction of pCIC that was imported at different KCl concentrations and found that, under these conditions, the percentage of imported pCIC vs bound pCIC decreased from 8.5% to 3% (Fig. 6F).

We therefore conclude that translocation of pCIC across the mitochondrial outer membrane of yeast mitochondria is inhibited by high salt concentrations because hydrophobic interactions of the pCIC presequence with the cytosolic domain of Tom20 are facilitated, thereby arresting pCIC at the mitochondrial outer surface.

The CIC we used in this study is a protein of mammalian (rat) cells. We next asked whether, in the presence of elevated ionic strength, import of pCIC into rat liver mitochondria is similarly reduced as the import into yeast mitochondria (Fig. 7A,B). Strikingly, with rat mitochondria we obtained almost identical results as with yeast mitochondria. Import of AAC was not affected by the KCl concentration, whereas import of Su9-DHFR clearly was. At a concentration of 480 mM KCl, translocation of Su9-DHFR into mitochondria was almost completely blocked (Fig. 7A). Import of mCIC was not significantly affected by increasing salt concentrations, but import of pCIC was again reduced (Fig. 7B). The interactions of the pCIC presequence appear to be very similar to mammalian and yeast import sites, both at physiological salt levels and at increasing salt concentrations.

Import of bovine phosphate carrier precursor protein (pPiC) is inhibited at increased ionic strength

Bovine pPiC carries a presequence of 49 residues that facilitates the import into mammalian mitochondria (Runswick et al., 1987; Zara et al., 1992). In previous studies, we observed that import of the protein into mitochondria of yeast was significantly inhibited by the presequence (Zara et al., 1992; Zara et al., 2003a). We now asked whether pPiC import is further inhibited at elevated salt concentrations. Using isolated mitochondria from *S. cerevisiae*, we imported pPiC at increasing KCl concentrations and determined the relative amounts of proteinase-K-protected protein (Fig. 7C,D). The pattern of salt-dependence showed that mPiC was imported with similar efficiencies at 80-480 mM KCl. By contrast, import of pPiC was strongly inhibited with increasing ionic strength. The result resembled the pattern that was observed

with the CIC. Almost the same inhibition was observed with rat liver mitochondria (data not shown). As in the case of pCIC, increased hydrophobic interactions of the pPiC presequence with components of the TOM complex appear to delay pPiC translocation across the mitochondrial outer membrane.

Discussion

Mitochondrial presequences were originally thought to primarily act as targeting signals. Consequently, efficient binding to import receptors was thought to be the essential task of these sequences (Schatz, 1987; Pfanner et al., 1991). The pCIC presequence demonstrates that both aspects, targeting and binding, are not necessarily essential in every case.

Many studies on mitochondrial protein import have already established that presequences of mitochondrial proteins can mediate several different functions (Roise and Schatz, 1988; Neupert, 1997; Rassow and Pfanner, 2000; Pfanner and Geissler, 2001; Rehling et al., 2003; Zara et al., 2003a): (1) Specific targeting of pre-proteins to mitochondria, (2) translocation of pre-proteins across the mitochondrial outer membrane, (3) targeting of the inner membrane TIM23 complex, (4) membrane-potential-dependent translocation across the inner membrane, (5) transfer of pre-proteins to the system of chaperone proteins in the mitochondrial matrix, and (6) sorting of pre-proteins to specific mitochondrial compartments. In a first study on the presequence of pCIC, we found that in this case the presequence seems not to act as a targeting signal but as a means of increasing the solubility of the hydrophobic pre-protein, thereby increasing the import competence of pCIC in the cytosol. The pCIC presequence appears to act similarly to an internal chaperone (Zara et al., 2003b). However, we cannot exclude that pCIC may modify the interactions of the pre-protein with the system of TOM and TIM complexes inside mitochondria, direct the CIC protein on a new import pathway, and thus participate in the functional aspects 2-6.

Based on the data presented in this study, we propose that under physiological conditions the presequence of rat pCIC is not engaged in any essential interactions with the mitochondrial protein import machinery. In the case of pCIC import, it is not the presequence that mediates the import of the mature protein into mitochondria but it is the mature protein that carries the presequence as a passenger peptide. The chaperoning function of the pCIC presequence seems to be the essential task in the biogenesis of the protein, demonstrating a further possible function of mitochondrial presequences in addition to the six functions mentioned above.

Whereas pCIC bypassed the import receptor Tom20 under normal conditions, it bound to Tom20 at increasing ionic strength. In this respect, the same observations were made with rat liver mitochondria (Fig. 1A and Fig. 7B) and with yeast mitochondria (Fig. 2A,B vs Fig. 6B), reflecting the structural similarities of Tom20 in all eukaryotes (Likic et al., 2005). The interaction was specifically dependent on the pCIC presequence because import of mCIC was completely independent of Tom20 at all salt concentrations tested. Surprisingly, the induction of interactions between the pCIC presequence and Tom20 did not confer an increased rate of import but a delay in membrane translocation. Inhibition of import at elevated salt concentrations was similarly observed with the presequence of the pPiC. In these cases, enhanced binding to receptor sites at the mitochondrial outer surface counteracts subsequent translocation. Enhanced binding of polypeptide chains at increasing ionic strength is commonly attributed to hydrophobic interactions (Dill, 1990), suggesting that the delay in translocation is due to lasting binding to the hydrophobic presequence binding site of Tom20 (Brix et al., 1997; Abe et al., 2000). Since positive charges are an essential element of mitochondrial presequences, salt-dependent inhibition of pre-protein import was traditionally thought to be a consequence of weakened ionic interactions (Pfaller et al., 1989; Haucke et al., 1995). Our study on the presequence of pCIC indicates that it is more likely that the inhibition is mainly the result of enhanced hydrophobic interactions.

Previous data demonstrating hydrophobic binding to Tom20 (Brix et al., 1997; Abe et al., 2000) were exclusively obtained with isolated proteins and questioned by other studies that were carried out with intact mitochondria. Several pre-proteins that expose hydrophobic targeting signals were found to either bypass Tom20 (Schneider et al., 1991; Ahting et al., 2005) or depend on a participation of positively charged residues in the vicinity of the hydrophobic segment (Motz et al., 2002; Stan et al., 2003). Mitochondrial import of porins is assisted by Tom20, although porins lack both a typical presequence and a hydrophobic membrane anchor (Krimmer et al., 2001; Müller et al., 2002). Our data on the import of pCIC are in agreement with the in vitro data (Brix et al., 1997; Abe et al., 2000) and support the conclusion that the same presequence binding site is accessible in isolated Tom20 and in mitochondrial Tom20 within the TOM complex.

The reactions that determine the rate-limiting steps in mitochondrial protein import have not been defined in detail. Several conditions were established that allow an arrest of translocating carrier proteins in distinct steps of the import pathway but the precise rate-limiting reactions under normal conditions are unknown. The results of the interaction of pCIC with the mitochondrial TOM machinery indicate that, at least in some cases, not only binding of pre-proteins to receptor sites but also their subsequent release can be rate-limiting.

Import of mitochondrial carrier proteins is mediated by a chain of binding sites (Ryan et al., 1999). An ‘acid-chain model’ was previously suggested to explain the import of hydrophilic pre-proteins (Schatz, 1997). In analogy to this model, a chain of hydrophobic binding sites may direct the import of carrier proteins. Three components that are directly involved in the passage of proteins across the outer membrane were suggested to act in a chaperone-like manner: Tom70 (Wiedemann et al., 2001), Tom40 (Esaki et al., 2003) and the Tim9/Tim10 complex (Koehler et al., 1998; Curran et al., 2002a; Vial et al., 2002; Lu et al., 2004). The observation of import inhibition by enhanced binding to receptor sites indicates that these components were evolutionary not optimised for the strongest binding of targeting signals but for specificity and the possibility of sufficiently rapid release. These requirements may account for the ambiguities that were observed, particularly in the characterization of Tom20. This import receptor shows specific interactions with several mitochondrial pre-proteins (Rapaport, 2003) but surprisingly low affinities to their targeting sequences (Brix et al., 1997; Brix et al., 1999; Abe et al., 2000). The bacterial chaperone protein GroEL revealed a system of structural changes to

release bound substrate proteins from hydrophobic binding sites (Hartl, 1996; Ranson et al., 1998; Sigler et al., 1998). It is tempting to speculate that the mitochondrial protein import machinery applies similar mechanisms to prevent irreversible binding to hydrophobic sites.

In summary, we propose three conclusions: (1) Differently from other mitochondrial pre-proteins, pCIC contains a presequence that does not act as a mediator of protein transport. The presequence is imported as a passenger of the mature protein, which contains the targeting information and does not interfere with the CIC import pathway. (2) The pCIC presequence binds to Tom20 when hydrophobic interactions are facilitated. (3) In general, mitochondrial outer membrane proteins can only act as import receptors if they show sufficiently rapid release of their substrate proteins. The stability of the receptor-substrate complex has to be low enough to allow a continuous flow within a sequence of binding sites.

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