

Nascent Polypeptide-associated Complex Stimulates Protein Import into Yeast Mitochondria

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To identify yeast cytosolic proteins that mediate targeting of precursor proteins to mitochondria, we developed an *in vitro* import system consisting of purified yeast mitochondria and a radio-labeled mitochondrial precursor protein whose C terminus was still attached to the ribosome. In this system, the N terminus of the nascent chain was translocated across both mitochondrial membranes, generating a translocation intermediate spanning both membranes. The nascent chain could then be completely chased into the mitochondrial matrix after release from the ribosome. Generation of this import intermediate was dependent on a mitochondrial membrane potential, mitochondrial surface proteins, and was stimulated by proteins that could be released from the ribosomes by high salt. The major salt-released stimulatory factor was yeast nascent polypeptide-associated complex (NAC). Purified NAC fully restored import of salt-washed ribosome-bound nascent chains by enhancing productive binding of the chains to mitochondria. We propose that ribosome-associated NAC facilitates recognition of nascent precursor chains by the mitochondrial import machinery.

INTRODUCTION

The great majority of mitochondrial proteins is encoded in the nucleus, synthesized on cytoplasmic ribosomes, and imported into mitochondria. Most matrix-targeted proteins carry an N-terminal, positively charged presequence that can form an amphiphatic α -helix and is usually cleaved by mitochondrial-processing peptidase (MPP) upon import into the matrix (Roise *et al.*, 1986; von Heijne, 1986). Precursor proteins are recognized on the mitochondrial surface by "TOM" receptors (for "translocase of the outer membrane") and translocated across the outer membrane through the TOM channel (Dekker *et al.*, 1998; Hill *et al.*, 1998; Künkele *et al.*, 1998). According to the "acid chain" hypothesis, the precursor is guided through the TOM channel by acidic binding sites on Tom proteins of increasing affinity for the positively charged presequence (Bolliger *et al.*, 1995; Dietmeier *et al.*, 1997; Schatz, 1997; Komiya *et al.*, 1998). The "TIM"-17/23 channel (for "translocase of the inner membrane") transports presequence-carrying precursors across

the inner membrane in a membrane potential-dependent way (Pfanner *et al.*, 1997). All precursors that are imported completely into the matrix require ATP in the matrix for the proposed import motor, mitochondrial hsp70 (Pfanner *et al.*, 1997). Some precursors also need ATP outside the mitochondria, probably for release from cytosolic chaperones (Wachter *et al.*, 1994).

Genetic and biochemical experiments suggest that cytosolic proteins are involved in mitochondrial protein import. Several genetic screens identified candidate genes in yeast, but the phenotypes of the mutations are often subtle and pleiotropic. Mutations of members of the SSA subfamily of cytosolic hsp70 proteins or of the cytosolic DnaJ homologue Ydj1p impair protein import into mitochondria and the endoplasmic reticulum (ER) *in vivo*, indicating a general role for these chaperones in both translocation systems (Deshaies *et al.*, 1988; Caplan *et al.*, 1992). A mutation in the cytosolic protein Mft52p (Cartwright *et al.*, 1997) or a deletion of the α -subunit of nascent polypeptide-associated complex (NAC) (George *et al.*, 1998) inhibit delivery of artificial fusion proteins, but not of authentic precursors, to mitochondria *in vivo*.

Biochemical studies in mammalian systems identified the ATPase "mitochondrial import stimulation factor" and cytosolic hsp70 proteins as cytosolic factors that keep precursors in an import-competent state and deliver them to the TOM receptors *in vitro*, thereby stimulating protein import into isolated mitochondria (Komiya *et al.*, 1996). Two additional cytosolic proteins, presequence binding factor (Murakami and Mori, 1990) and targeting factor (Ono and Tuboi,

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Abbreviations used: ER, endoplasmic reticulum; MPP, mitochondrial processing peptidase; NAC, nascent polypeptide-associated complex; RNC, ribosome-nascent chain complex; SRP, signal recognition particle; TCA, trichloroacetic acid; TIM, translocase of the inner membrane; TOM, translocase of the outer membrane.

1990), were reported to stimulate protein import into isolated mammalian mitochondria, but neither the amino acid sequence nor the mechanism of action of these proteins is known.

To identify yeast cytosolic "import stimulation factors" and to study the mechanism of the early steps of mitochondrial protein import, we set up a fully homologous *in vitro* import assay in that cytosol, mitochondria, and precursor were derived from yeast to avoid artifacts arising from heterologous combinations. *In vivo*, fully synthesized but unprocessed mitochondrial precursor proteins are almost undetectable (Fujiki and Verner, 1993), suggesting that their import is extremely fast and occurs either cotranslationally (Ades and Butow, 1980) or very soon after synthesis is complete. In both cases, import-stimulating factors are likely to associate with the nascent chain still bound to the ribosome. To identify such import-stimulating factors we have translated the precursor protein *in vitro* from an mRNA lacking the stop codon, thereby generating ribosome-nascent chain complexes (RNCs) in which the C terminus of the nascent chain remains bound to the ribosome (Mueckler and Lodish, 1986; Gilmore *et al.*, 1991). RNCs have two major advantages over released precursor chains: 1) they can be separated from the bulk cytosol by sedimentation through a sucrose cushion, which allows supplementation with selected cytosolic factors during the import reaction; and 2) the precursor protein remains "trapped" in a "nascent conformation," which prevents folding and might stabilize interactions with specific chaperones or targeting factors. An analogous approach has been a key to understanding the mechanisms of protein translocation into the ER (Gilmore *et al.*, 1991).

Upon incubation of isolated RNCs with purified mitochondria, the N terminus of the nascent chain was imported into the matrix and processed by MPP, whereas the C terminus was still attached to the ribosome on the mitochondrial surface. Yeast NAC was identified as a factor that stimulated formation of this two-membrane spanning import intermediate by enhancing productive binding of the nascent chains to the mitochondria.

MATERIALS AND METHODS

Purification of NAC

A 24-l culture of the protease-deficient yeast strain C13 ABYS-86 (Heinemeyer *et al.*, 1991) was grown at 30°C in YPD medium overnight to an OD₆₀₀ of 1.2. Cells (120 g wet weight) were harvested and converted to spheroplasts by Zymolyase (Seikagaku America, Falmouth, MA) treatment. The spheroplasts were washed in 2.4 l of sorbitol buffer (1.4 M sorbitol, 50 mM K_{P_i}, pH 7.4, 5–10 mM DTT) and lysed in 200 ml of lysis buffer (20 mM HEPES, pH 7.4, 100 mM K-acetate, pH 7.4, 2 mM Mg-acetate, 2 mM DTT, 0.5 mM PMSF, 1.25 μg/ml leupeptin, 0.75 μg/ml antipain, 0.25 μg/ml chymostatin and elastinol, 5 μg/ml pepstatin) in an all-glass Dounce homogenizer. The homogenate was cleared by centrifugation for 15 min at 27,000 × *g* and then 30 min at 80,000 × *g*. The clear supernatant (140 ml) was layered on top of 2 vol of buffer S1 (25% sucrose, 20 mM HEPES, pH 7.4, 120 mM K-acetate, pH 7.4, 5 mM Mg-acetate, 2 mM DTT, 0.5 mM PMSF) and centrifuged for 2.5 h at 160,000 × *g* to sediment the ribosomes. The ribosomal pellet was resuspended in buffer S1 containing 0.7 M K-acetate using a Dounce homogenizer with a Teflon piston, and the suspension was centrifuged for 2.5 h at 160,000 × *g*. The supernatant was saved and diluted with 3.5 vol of buffer A (buffer S1 lacking sucrose and K-acetate), cleared by

centrifugation for 20 min at 1200 × *g*, and loaded onto a ResourceQ anion exchange column (Pharmacia, Piscataway, NJ). Bound proteins were eluted with a linear 80 ml K-acetate gradient (200–600 mM) and NAC eluted at ~440 mM K-acetate. NAC-containing fractions were pooled, diluted with 0.33 vol of buffer A, and loaded onto a MonoQ anion exchange column (Pharmacia). Bound proteins were eluted with a linear 60-ml K-acetate gradient (500–700 mM) and NAC eluted at ~620 mM K-acetate. NAC-containing fractions were pooled, diluted with 1 vol of buffer A, concentrated in a Centricon-30 device (Millipore, Bedford, MA), frozen in small aliquots in liquid nitrogen, and stored at –80°C.

Import Assay

Mitochondria were isolated from the protease-deficient yeast strain C13 ABYS-86 (Heinemeyer *et al.*, 1991) and purified on a Nycodenz gradient (Nycodenz Pharma, Oslo, Norway) (Glick and Pon, 1995). For the study of the receptor mutants, the yeast strains deleted either in TOM70 (YVH1) (Hines and Schatz, 1993) or TOM20 (YTJB64) (Lithgow *et al.*, 1994b) and a corresponding wild-type strain (JKR101) were grown on YPGal (1% yeast extract, 1% peptone, 2% galactose), because YTJB64 grows poorly on nonfermentable carbon sources. Crude mitochondria were prepared according to the method of Glick and Pon (1995), except that the last purification step on the Nycodenz gradient was omitted. Import reactions contained, unless stated otherwise, 0.8 mg/ml mitochondria in "import buffer" (0.32 mg/ml BSA, 20 mM HEPES, pH 7.4, 120 mM K-acetate, pH 7.4, 5 mM Mg-acetate, 0.6 M sorbitol, 0.05 U/μl RNase inhibitor [from human placenta], and 2 mM DTT) supplemented with 2 mM ATP, NADH, and K_{P_i}. The mitochondria were premixed with import buffer, and imports were started by the addition of 12.5% (vol/vol) RNCs (see below) and were carried out at 20°C for up to 20 min. Volumes of import varied from 100 to 500 μl. At the indicated time points, 100-μl samples were withdrawn for further analysis. Protease treatment to probe for import and folding was performed as described (Rospert and Schatz, 1998).

Construction of Truncated MDH1

The yeast mitochondrial MDH1 gene lacking the stop codon was amplified with Pfu polymerase from yeast genomic DNA using the primers MDH-F (ggcgctgctgggatccatgttgcaagagtagct) and MDH-R2 (ctgcagttactagcaacaagttagacc). The PCR product and the expression plasmid pSP65 (Promega, Madison, WI) were cut with *Bam*HI and *Pst*II, ligated, and transformed into the *Escherichia coli* strain DH10B. The resulting plasmid is termed pSP65-MDHt.

In Vitro Translation and Preparation of RNCs

Yeast translation extract was prepared as described (Garcia *et al.*, 1991) from the protease-deficient strain C13-ABYS-86 (Heinemeyer *et al.*, 1991). mRNA for truncated Mdh1p was generated by *in vitro* transcription using SP6 polymerase from plasmid pSP65-MDHt, phenol-chloroform extracted, ethanol precipitated, resuspended in water, and stored in aliquots at –80°C. Translations were performed as described (Garcia *et al.*, 1991) at 20°C for 30 min and cleared by centrifugation for 10 min at 20,000 × *g* at 4°C. RNCs were pelleted by centrifugation through two volumes of buffer S2 (25% sucrose, 20 mM HEPES, pH 7.4, 5 mM Mg-acetate, 2 mM DTT, 0.005 U/μl RNase inhibitor [from human placenta]) containing either 120 mM K-acetate (untreated RNC) or 0.7 M K-acetate (salt-washed RNC) at 200,000 × *g* in a TLA-100 tabletop ultracentrifuge (Beckman Instruments, Palo Alto, CA) for 50 min at 2°C. The clear pellets were resuspended in import buffer lacking BSA in a volume equal to the original translation reaction.

Solubilization of Mitochondria and Isolation of RNCs

Import was performed as described for 20 min at 20°C and stopped by transfer on ice, supplemented with protease inhibitors (1 mM

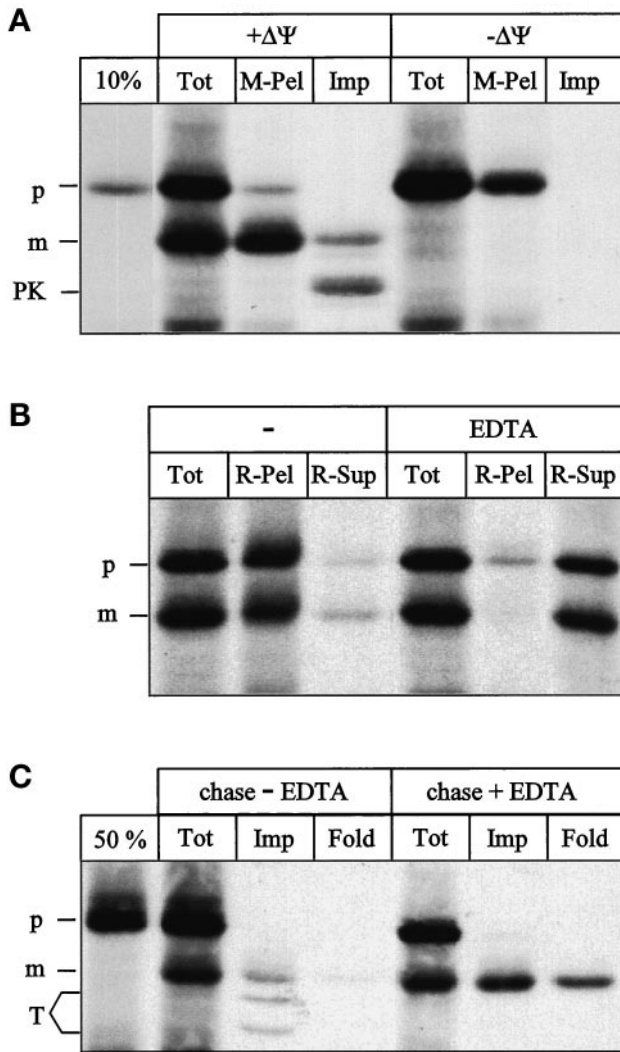


Figure 1. Ribosome-associated nascent chains can generate a mitochondrial import intermediate spanning both membranes. (A) Generation of the import intermediate. RNCs carrying the radiolabeled Mdh1p precursor were isolated and incubated with yeast mitochondria as described in MATERIALS AND METHODS for 20 min at 20°C in the absence (+ΔΨ) or presence (-ΔΨ) of 1 μg/ml valinomycin. Equal aliquots were precipitated with TCA (Tot), centrifuged for 3 min at 14,000 × g to recover the mitochondria (M-Pel), or treated with 100 μg/μl proteinase K for 15 min on ice, followed by inactivation of the protease with 1 mM PMSF, reisololation of the mitochondria, and precipitation with TCA (Imp). All samples were analyzed by SDS-PAGE and fluorography. 10%, 10% of the RNCs added per lane. (B) The intermediate is ribosome attached. Import was performed as in A and stopped by transfer to 0°C. The sample was then split into two equal aliquots. The mitochondria in both aliquots were solubilized in 1% Triton X-100 either in the presence (EDTA) or absence (-) of 10 mM EDTA as described in MATERIALS AND METHODS. One-half of each aliquot was precipitated with TCA (Tot); the other half was separated into a ribosomal pellet (R-Pel) and a supernatant (R-Sup). All samples were analyzed by SDS-PAGE and fluorography. (C) Chase of the intermediate. Import was performed as in A, and further generation of the import intermediate was stopped after 20 min by the addition of 1 μg/ml valinomycin. The reaction was split into two equal aliquots. One

PMSF, 1.25 μg/ml leupeptin, 0.75 μg/ml antipain, 0.25 μg/ml chymostatin and elastinol, 5 μg/ml pepstatin) and Triton X-100 to a final concentration of 1% in the presence or absence of 10 mM EDTA. Samples were left on ice for 10 min and cleared by centrifugation for 10 min at 14,000 × g at 4°C. The supernatants were layered on top of 2 vol of buffer S2 containing 120 mM K-acetate and 1% Triton X-100. RNCs were sedimented at 200,000 × g for 40 min at 4°C.

Preparation of Salt Wash Fluid and Immunodepletion

For the preparation of salt wash fluid, ribosomes from yeast translation extract were pelleted by centrifugation through 2 vol of buffer S2 containing 120 mM K-acetate for 50 min at 200,000 × g at 2°C. The ribosomes were resuspended in buffer S2 containing 0.7 M K-acetate and pelleted again for 50 min at 200,000 × g at 2°C. The resulting supernatant was the salt wash fluid. Immunoprecipitations were performed on ice for 2.5 h in 20 mM HEPES, pH 7.4, 120 mM K-acetate, pH 7.4, 5 mM Mg-acetate, 0.5 mM PMSF, 1.25 μg/ml leupeptin, 0.75 μg/ml antipain, 0.25 μg/ml chymostatin and elastinol, 5 μg/ml pepstatin; using 3 μl of polyclonal rabbit serum/1 μl of swollen protein A-Sepharose beads.

MPP Assay

Recombinant MPP was a gift from P. Luciano and V. Géli (LISM-CRNS, Marseille, France) (Luciano *et al.*, 1998). RNCs were resuspended in import buffer and supplemented with 0.04 μg/μl NAC as indicated. MPP was added to 0.5 μM, and the samples were incubated at 20°C. After the indicated times, 30-μl aliquots were withdrawn and precipitated with trichloroacetic acid (TCA). Cleavage was assayed by SDS-PAGE and fluorography.

Miscellaneous

Protein concentrations were determined by the Bradford assay (from Bio-Rad, Hercules, CA) using BSA as a standard. ¹²⁵I-labeled protein A was used to develop the immunoblots.

RESULTS

Ribosome-attached Nascent Chains Form Productive Import Intermediates

We chose the precursor of yeast mitochondrial malate dehydrogenase (Mdh1p) as a model protein, because it carries a typical N-terminal, cleavable presequence and folds into a protease-resistant conformation inside mitochondria (Duba-

Figure 1 (cont). aliquot was left untreated at 20°C (chase - EDTA); the other aliquot was treated with 10 mM EDTA for 10 min at 20°C, followed by the addition of 10 mM MgAcetate (chase + EDTA). One-third of each aliquot was precipitated with TCA (Tot); the remaining two-thirds were treated with 100 μg/ml trypsin for 30 min on ice. After inactivation of the protease with 200 μg/ml soy bean trypsin inhibitor and reisololation of the mitochondria, the trypsin-treated mitochondria were subdivided in two equal samples. The first sample was precipitated with TCA (Imp), and the second sample was treated with 1% Triton X-100 plus 100 μg/ml proteinase K for 15 min on ice. After inactivation of the protease with 1 mM PMSF, the second sample was also precipitated with TCA (Fold). All samples were analyzed by SDS-PAGE and fluorography. 50%, 50% of the RNCs added per lane; p, Mdh1p precursor; m, mature Mdh1p; PK, proteinase K-protected fragment; T, trypsin-protected fragments.

quié *et al.*, 1998). When the Mdh1p precursor was translated from an mRNA lacking a stop codon, the C terminus of the full-length precursor of 335 amino acids remained attached to the ribosome, forming a stable RNC (Figure 1B) (Mueckler and Lodish, 1986; Gilmore *et al.*, 1991).

To test whether the ribosome-attached precursor chain could engage the mitochondrial import machinery, RNCs were isolated by centrifugation through a sucrose cushion and incubated with isolated yeast mitochondria (Figure 1A).

Approximately 45% of the chains were processed to the mature size (Tot), indicating that their N-terminus had reached the matrix. Import was dependent on a potential across the mitochondrial inner membrane (Tot, $-\Delta\Psi$). All processed chains, but very few unprocessed ones, were recovered in the mitochondrial pellet (M-Pel). After treatment of the mitochondria with proteinase K, only 14% of mature-sized chains was fully protected; the bulk was converted to a smaller protected fragment (Imp), indicating that a portion of the chain was accessible from outside. To test whether the processed chains were still attached to ribosomes, we isolated the RNCs from a total import reaction by solubilizing the mitochondria in 1% Triton X-100 and sedimentation through a sucrose cushion (Figure 1B). Triton X-100 solubilizes membranes but leaves ribosomes intact (Schneider *et al.*, 1976). The majority of both precursor and mature Mdh1p was recovered in the ribosomal pellet (R-Pel), indicating that they were still attached to ribosomes. Solubilization in the presence of 10 mM EDTA dissociated the ribosomes and released most of the processed as well as unprocessed Mdh1p into the supernatant (Figure 1B, EDTA). Ribosome-attached nascent chains are thus not completely imported but accumulate as processed, two-membrane-spanning import intermediates.

To test whether these import intermediates could be chased into the matrix, we accumulated them as described in Figure 1A, stopped further accumulation by addition of the uncoupler valinomycin, and split the reaction in two. One-half was left untreated, and the other half was treated with EDTA to release the nascent chain from the ribosome. Both halves were then "chased" for 10 min (Figure 1C). When the chase was conducted in the absence of EDTA, one-third of the chains were processed to the mature size (Tot), and trypsin generated mature-sized chains as well as two smaller protected fragments (Imp). The pattern of protease-protected fragments depended on the type of protease used (Figure 1, compare A and C, Imp lanes). The protected fragments and most of the mature chains were not properly folded, because they were sensitive to proteinase K after solubilization of the mitochondria with Triton X-100 (Fold). When the chase was conducted in the presence of EDTA, 70% of the mature chains were protease-protected in intact mitochondria (Imp), and 34% had folded into a protease-resistant conformation (Fold). Ribosome-attached precursor chains can thus form productive import intermediates that can be completely imported into mitochondria upon release from the ribosome.

To test whether proteins peripherally associated with RNCs influenced the import of ribosome-attached chains, we prepared salt-washed RNCs by centrifugation through a sucrose cushion containing 0.7 M K-acetate. Salt washing of RNCs greatly reduced the import efficiency of the attached precursor chains (Figure 2).

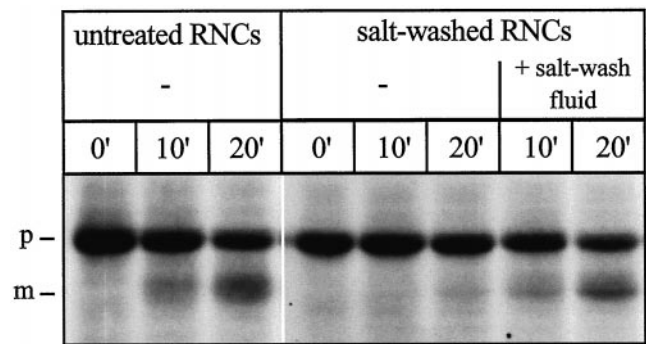


Figure 2. Salt washing of RNCs reversibly reduces import of the attached nascent chains. Untreated RNCs, salt-washed RNCs, and a ribosomal salt wash fluid were prepared as described in MATERIALS AND METHODS. Salt wash fluid was prepared from an amount of ribosomes corresponding to the amount of ribosomes present as RNCs in the import reaction. Import reactions were performed with untreated RNCs, salt-washed RNCs, and salt-washed RNCs mixed with salt wash fluid (+ salt-wash fluid) as described in MATERIALS AND METHODS. After the indicated times, equal samples were precipitated with TCA and analyzed by SDS-PAGE and fluorography. p, Mdh1p precursor; m, mature Mdh1p.

This effect was reversible, readdition of a ribosomal salt wash fluid corresponding to the amount of proteins released from the RNCs, almost completely restored import. Heating of the salt wash fluid to 95°C for 5 min or protease treatment abolished the stimulatory activity, suggesting it is caused by a protein (our unpublished data).

Identification of the Stimulating Factor

We fractionated a ribosomal salt wash fluid by anion exchange chromatography and tested the fractions for import stimulation of ribosome-attached nascent chains. The active fractions contained equal amounts of two prominent proteins with apparent molecular masses of 28 and 21 kDa. By mass spectrometry of tryptic fragments, the two proteins were identified as the α - and β -subunits of yeast NAC. This complex has been described both in yeast and in mammals, although in a different context (Parthun *et al.*, 1992; Wiedmann *et al.*, 1994; Shi *et al.*, 1995; George *et al.*, 1998).

To verify that NAC acts indeed as an import stimulatory factor, a ribosomal salt wash fluid was either mock depleted with preimmuneserum or immunodepleted with immuneserum against yeast α NAC (George *et al.*, 1998). After immunodepletion, α NAC was undetectable in the salt wash fluid and quantitatively recovered on the beads (Figure 3A).

The import stimulatory activity of α NAC-depleted salt wash fluid was reduced to 20% compared with the mock-depletion (Figure 3B). Consistent with published data (Parthun *et al.*, 1992; Wiedmann *et al.*, 1994; Reimann *et al.*, 1999), we identified α - and β NAC as a stable complex and the purified complex could be quantitatively immunoprecipitated with immuneserum against α NAC (Figure 4 and our unpublished data). The complex of α - and β NAC thus constitutes the major stimulatory activity in the salt wash fluid.

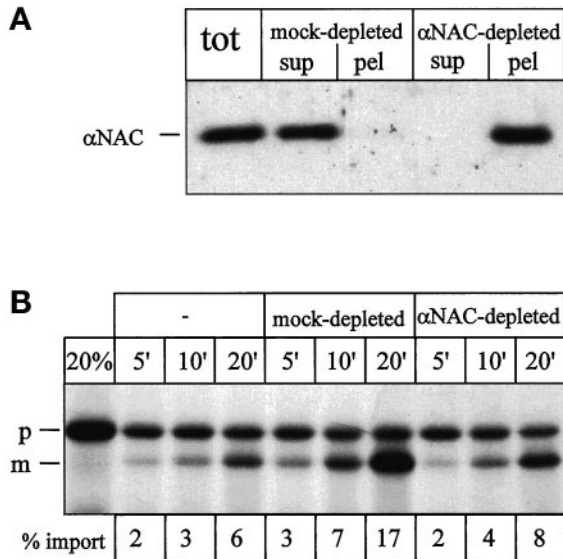


Figure 3. Immunodepletion of NAC from salt wash fluid depletes most but not all import stimulatory activity. (A) Immunodepletion of salt wash fluid. Equal aliquots of salt wash fluid (see Figure 2) were either left untreated (tot), mock depleted with preimmuneserum (mock-depleted), or immunodepleted with immuneserum against α NAC (α NAC-depleted). The treated aliquots were separated into supernatant (sup) and beads (pel), and all samples were analyzed for α NAC by SDS-PAGE and immunoblotting. (B) Import stimulation by salt wash fluid. Import reactions were performed with salt-washed RNCs without addition (-), after addition of mock-depleted salt wash fluid (mock-depleted), or after addition of α NAC-depleted salt wash fluid (α NAC-depleted) as described in MATERIALS AND METHODS. After the indicated times, equal samples were centrifuged for 3 min at $14,000 \times g$ to recover the mitochondria. All samples were analyzed by SDS-PAGE and fluorography and quantified by densitometry. % import, percentage of mature Mdh1p given as fraction of initially added Mdh1p; 20%, 20% of the RNCs added per lane; p, Mdh1p precursor; m, mature Mdh1p.

After refinement of the fractionation scheme used to identify NAC, we purified NAC by a factor of 900 from total yeast cytosol (Figure 4 and Table 1).

Purified NAC stimulated mitochondrial import of precursor chains on salt-washed RNCs in a dose-dependent manner from 10 up to 56% (Figure 5).

Untreated RNCs containing endogenous NAC at concentrations of $0.06 \mu\text{g}/100 \mu\text{l}$ of import reaction were translocated with an efficiency of 20%. To restore import efficiency of salt-washed RNCs to 20%, $0.24 \mu\text{g}$ of our purified NAC preparation was required per $100\text{-}\mu\text{l}$ import reaction. A larger excess of purified NAC stimulated import to a significantly greater extent than the pool of factors bound to untreated RNCs (compare DISCUSSION).

Accessibility of the Precursor to Added MPP

A possible explanation for the low import efficiency of salt-washed RNCs would be that the positively charged presequence folds back onto the negatively charged ribosome and cannot be recognized by the TOM receptors on the mito-

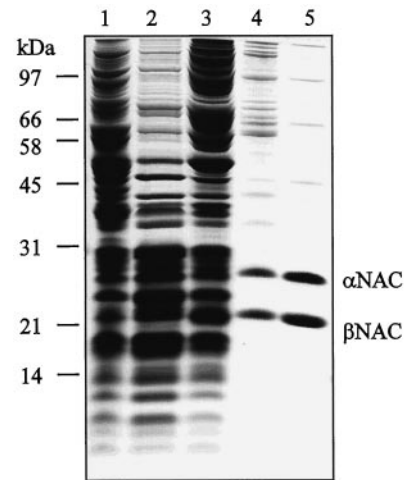


Figure 4. Purification of NAC from yeast cytosol. Yeast NAC was purified as described in MATERIALS AND METHODS, and the following fractions were analyzed by SDS-PAGE and staining with Coomassie blue: lane 1, cytosol; lane 2, ribosomal pellet; lane 3, salt wash fluid; lane 4, pooled fractions after ResourceQ chromatography; lane 5, pooled fractions after MonoQ chromatography. Lanes 1-3 contain $100 \mu\text{g}$ of protein; lanes 4 and 5 contain $10 \mu\text{g}$ of protein. Molecular mass standards are indicated on the left.

chondrial surface. To probe the accessibility of the presequence, untreated and salt-washed RNCs were incubated with purified yeast MPP (Luciano *et al.*, 1998) in the presence or absence of NAC (Figure 6).

The precursor on untreated RNCs was cleaved only slowly, suggesting that it is relatively inaccessible. Addition of concentrations of purified NAC that fully stimulated translocation, decreased the cleavage rate even further. In contrast, the precursor on salt-washed RNCs was more accessible to MPP. Addition of purified NAC did not restore protection but increased the cleavage rate even further. We conclude that the MPP cleavage site on ribosome-associated nascent chains is protected by salt-extractable factors other than NAC, and that the presequence on salt-washed RNCs is accessible to surface receptors on the mitochondria.

NAC Stimulates Productive Binding of RNCs to Protease-sensitive Sites on Mitochondria

To test the influence of mitochondrial surface proteins on import stimulation, we compared import of nascent chains into trypsin-shaved or mock-treated mitochondria (Figure 7). Pretreatment of the mitochondria with $100 \mu\text{g}/\text{ml}$ trypsin for 5 min on ice completely removed the protease-sensitive cytosolic domains of the receptors Tom70p, Tom20p, and Tom22p, whereas the protease-sensitive inner membrane protein Tim23p remained intact (Figure 7A, WT). Salt-washed RNCs were incubated with trypsin or mock treated mitochondria in the presence or absence of purified NAC (Figure 7B, WT).

In mock-treated wild-type mitochondria, NAC stimulated import 2.5-fold. After pretreatment of the mitochondria with trypsin, import stimulation by NAC was almost abolished, indicating that it depends on trypsin-sensitive mitochon-

Table 1. Purification of NAC and the import-stimulatory activity from yeast cytosol

Fraction	Total protein (mg)	NAC (mg) ^a	Yield of NAC (%)	Purification of NAC (fold)	Total activity (U) ^b	Yield of activity (%)	Specific activity (U/mg)	Purification of activity (fold)
Cytosol	2,200	2.5	100	1	121,000	100	55	1
Ribosomal pellet	380	2.0	83	5	21,600	18	57	1
Salt wash fluid	38	0.63	25	15	20,000	17	530	10
ResourceQ column eluate	1.70	0.51	20	272	4,688	4	2,800	50
MonoQ column eluate	0.62	0.62	25	892	2,530	2	4,000	74

^a Estimated by quantitative immunoblotting using purified NAC as a standard.

^b One unit is defined as the activity that doubles import of Mdh1p bound to salt-washed RNCs into isolated mitochondria under the import conditions specified in MATERIALS AND METHODS.

drial surface proteins (Figure 7B, WT). The two best studied receptors on the outer mitochondrial membrane, Tom70p and Tom20p, show differential specificities in posttranslational precursor recognition (Lithgow *et al.*, 1995; Lill and Neupert, 1996). To test the influence of Tom70p and Tom20p on NAC-dependent import of nascent chains, we compared import into mitochondria prepared from a wild-type strain (WT), a strain carrying a deletion of TOM70 (Δ TOM70), and a strain carrying a deletion in TOM20 (Δ TOM20) (Figure 7B) (Hines and Schatz, 1993; Lithgow *et al.*, 1994b). The deletion mutations were confirmed by immunoblotting for Tom70p and Tom20p (Figure 7A). Stimulation of translocation in the presence of NAC was independent of Tom20p or Tom70p, indicating that these two proteins are not responsible for the trypsin sensitivity of NAC stimulation (Figure 7B).

In wild-type and Δ TOM70 mitochondria, residual NAC-independent import was not affected by trypsin-treatment. However, it was almost abolished in Δ TOM20 mitochondria. Possibly, the protease-resistant fragment of Tom20p (Figure

7A, Tom20*) is sufficient for NAC-independent import in the other two strains. Alternatively, other components of the TOM complex, e.g., Tom22p, might be destabilized and more easily degraded in Δ TOM20 mitochondria (Figure 7A, Tom22*) (Lithgow *et al.*, 1994a).

To determine the stage of import that was stimulated by NAC, we performed a binding and chase experiment. Salt-washed RNCs were first incubated with nonenergized mitochondria on ice in the presence or absence of NAC (Figure 8) to allow binding of RNCs.

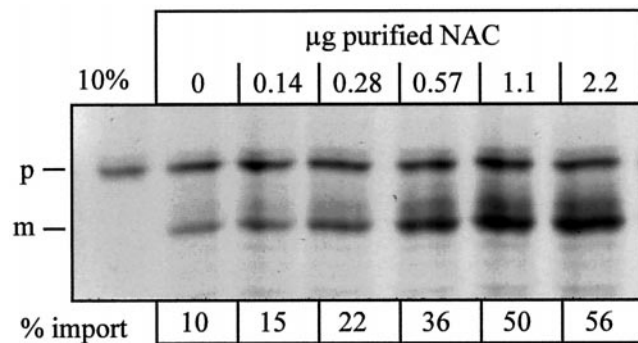


Figure 5. Purified NAC stimulates import of ribosome-associated nascent chains into mitochondria. Import reactions were performed with salt-washed RNCs in the presence of the indicated amounts of purified NAC in a total volume of 100 μ l as described in MATERIALS AND METHODS. After 15 min of import, all samples were centrifuged for 3 min at 14,000 \times g to recover the mitochondria and were analyzed by SDS-PAGE and fluorography. The resulting bands on the fluorogram were quantified by densitometry, and the percentage of processed chains was determined, with the amount of initially added Mdh1p set to 100% (% import). 10%, 10% of the RNCs added per lane; p, Mdh1p precursor; m, mature Mdh1p.

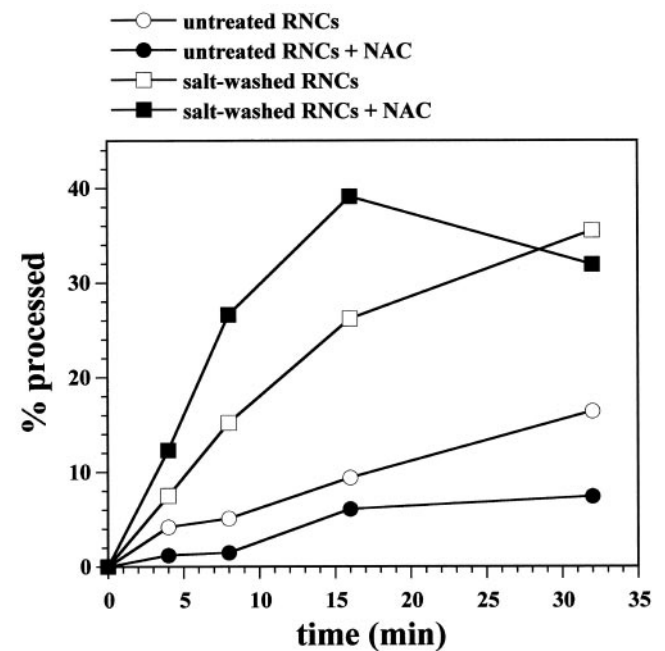


Figure 6. Salt washing of RNCs increases the accessibility of the attached nascent chains to MPP *in vitro*. Untreated and salt-washed RNCs were incubated in import buffer with 0.5 μ M purified MPP in the presence or absence of 40 μ g/ml purified NAC at 20°C for the indicated times. Cleavage of the Mdh1p precursor was analyzed by SDS-PAGE and fluorography. The percentage of cleaved chains was determined by densitometry of the fluorogram, with the amount of initially added Mdh1p set to 100%.

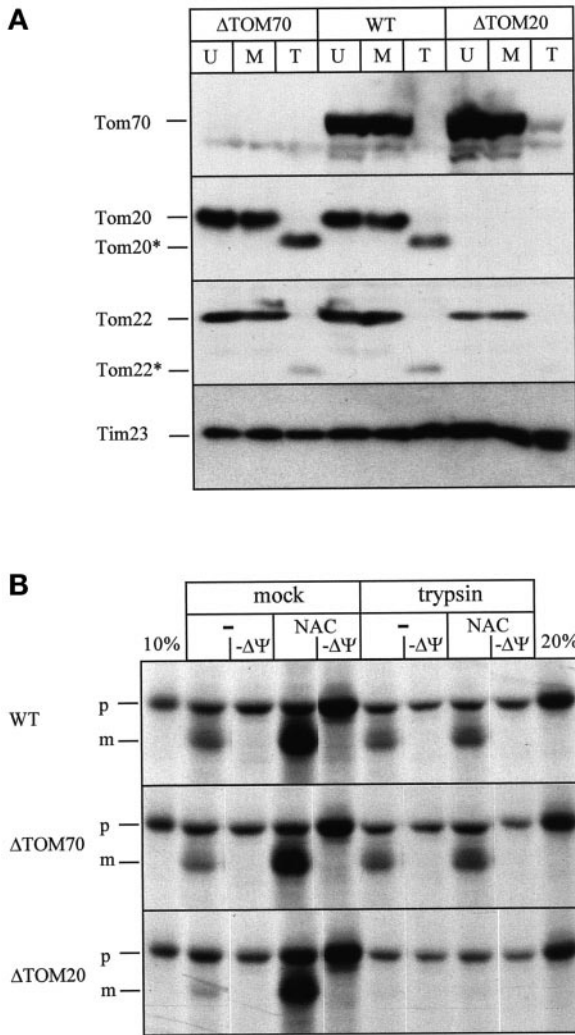


Figure 7. Stimulation of import by NAC depends on mitochondrial surface proteins. (A) Trypsin treatment of mitochondria. Three aliquots of crude mitochondria from yeast strains YVH1 (Δ TOM70), JKR101 (WT), or YVH1 (Δ TOM20) each were treated as follows. The first aliquot was left untreated on ice (U); the second aliquot was treated with 100 μ g/ml trypsin for 5min on ice, followed by inactivation of trypsin with 200 μ g/ml soybean trypsin inhibitor for 5 min and reisolation of the mitochondria (T); and the third aliquot was mock-treated with 100 μ g/ml trypsin that had been inactivated by preincubation with 200 μ g/ml trypsin inhibitor for 5 min on ice followed by reisolation of the mitochondria (M). Equal amounts of mitochondrial protein (75 μ g) were analyzed by SDS-PAGE and immunoblotting for Tom70p, Tom20p, Tom22p, and Tim23p as indicated on the side. Tom20* and Tom22* denote a trypsin-generated fragment of Tom20p and Tom22p, respectively. (B) Mitochondrial surface proteins are required for import stimulation by NAC. Imports were performed with the same trypsin-treated (trypsin) or mock-treated (mock) mitochondria as shown in A with salt-treated RNCs in the presence (NAC) or absence (-) of 10 μ g/ml NAC. The strain is indicated on the left. After 7.5 min, mitochondria were recovered by centrifugation for 3 min at 14,000 \times g, and analyzed by SDS-PAGE and fluorography. 10 and 20%, 10 and 20% of the RNCs added per lane, respectively; $-\Delta\Psi$, import in the presence of 1 μ g/ml valinomycin and 25 μ M carbonyl cyanide *p*-trifluoromethoxyphenyl-hydrazone; p, Mdh1p precursor; m, mature Mdh1p.

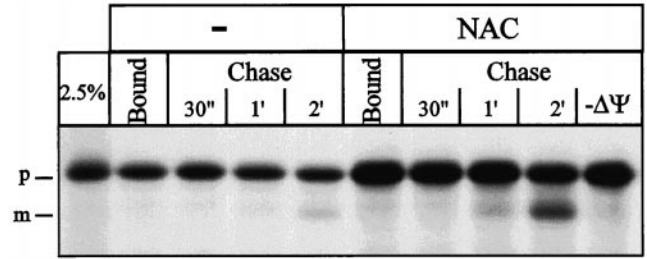


Figure 8. NAC promotes productive binding of RNCs to mitochondria. Salt-washed RNCs were incubated with 0.15 mg/ml mitochondria in import buffer for 5 min on ice in the presence (NAC) or absence (-) of 11 μ g/ml NAC. The mitochondria were reisolated, resuspended in import buffer, and divided in two aliquots. One aliquot was precipitated with TCA (bound). The other aliquot was supplemented with 2 mM ATP, NADH, and KP_i and shifted to 20°C. After the indicated chase times, samples were withdrawn and precipitated with TCA and analyzed by SDS-PAGE and fluorography. $-\Delta\Psi$, chase for 2 min in the presence of 1 μ g/ml valinomycin; 2.5%, 2.5% of the RNCs added per lane; p, Mdh1p precursor; m, mature Mdh1p.

Reisolation of the mitochondria revealed that NAC stimulated binding of RNCs to mitochondria twofold (Bound). Bound precursor chains were chased into the mitochondria by energizing the mitochondria and raising the temperature to 20°C. After 2 min, only 6% of the chains that had bound to mitochondria in the absence of NAC were imported, whereas 23% were imported after binding in the presence of NAC (Chase). Addition of NAC during the chase did not influence import (our unpublished data). NAC thus stimulates productive binding of RNCs to mitochondria when bound to RNCs before their contact with the mitochondrial surface. If formation of a membrane potential was prevented by the addition of valinomycin after the binding step, but before the chase, import was completely inhibited (Figure 8; Chase, $-\Delta\Psi$). Incubation of RNCs with nonenergized mitochondria on ice thus only allows binding but not import of the attached precursor chains.

DISCUSSION

Purification of NAC as an Import Stimulatory Factor

Using a novel in vitro assay for the study of the initial steps of mitochondrial protein import in vitro, we have identified yeast NAC, a complex of α - and β -subunits, as a factor that stimulates import of ribosome-associated nascent chains into mitochondria. Yeast NAC was originally described in an entirely different context as a complex of two nonessential proteins that stabilizes the binding of the transcriptional activator Gal4p to DNA (Parthun *et al.*, 1992; Shi *et al.*, 1995), suggesting a function in the nucleus. The individual subunits of mammalian NAC have also been described as factors involved in transcription (Zheng *et al.*, 1990; Moreau *et al.*, 1998; Yotov *et al.*, 1998). In addition, and consistent with our findings, Wiedmann *et al.* (1994) identified mammalian NAC as a ribosome-associated complex that can be cross-linked to short nascent chains destined to various compartments of the cell. NAC was proposed to enhance targeting

idelity by preventing promiscuous interaction of signal recognition particle (SRP) with nascent chains not destined for the ER (Wiedmann *et al.*, 1994). Alternatively NAC might modulate the interaction of SRP with the ribosome (Powers and Walter, 1996) or the interaction of RNCs with the ER (Lauring *et al.*, 1995a,b; Möller *et al.*, 1998). The latter finding has been challenged (Neuhof *et al.*, 1998; Raden and Gilmore, 1998). Recent experiments describe yeast NAC as a ribosome-associated complex involved in mitochondrial protein import and possibly targeting to the ER (George *et al.*, 1998). As will be discussed below, NAC shows a stimulatory effect on mitochondrial protein import, whereas it acts as a negative control element during import into the ER.

NAC Alone Does Not Shield the Nascent Chain against Purified MPP In Vitro

Mammalian MDH is cleaved in a two-step process involving both MPP and intermediate processing peptidase (Shimokata *et al.*, 1997). The same was proposed for yeast Mdh1p (Branda and Isaya, 1995). We observed that Mdh1p was indeed cleaved to an intermediate form by recombinant MPP (our unpublished data), suggesting that MPP cleaves off the first 9 amino acids of the 17-amino acid-long presequence.

Salt washing of RNCs enhanced presequence cleavage by purified MPP (Figure 6), and concentrations of NAC that stimulated import efficiently did not restore protection against MPP. This result suggests that additional factors are bound to untreated RNCs and protect the presequence part of the nascent chain. These factors, however, are dispensable for import in our system, because purified NAC fully restores import of salt-washed RNCs. Wang *et al.* (1995) have shown that NAC was both necessary and sufficient to protect short nascent chains (up to 44 amino acids) against proteolysis. This is in good agreement with the finding that NAC can be cross-linked to very short nascent chains and probably is in close contact to the exit site of the nascent chain on the ribosome (Wiedmann *et al.*, 1994). We used a nascent chain of 335 amino acids, which was too long for the N-terminal presequence to be protected by NAC. The accessibility of the nascent chains to MPP, as well as the reversibility of the import competence, suggest that the nascent chain on salt-washed RNCs does not irreversibly aggregate or adhere to the ribosomal surface.

Mode of Action of NAC

NAC stimulated productive binding of RNCs to mitochondria. The first recognition of precursor proteins on the outer mitochondrial membrane is thought to be mediated by Tom20p and by Tom70p, two receptors that exhibit different specificities for mitochondrial precursor proteins. Tom20p binds preferentially to the charged presequence of the precursor protein, whereas Tom70p binds more tightly to internal sequences (Moczko *et al.*, 1994; Komiya *et al.*, 1998; Brix *et al.*, 1999). As a consequence, productive binding of presequence-containing precursor proteins, such as Mdh1p, to the mitochondrial surface is predominantly mediated by Tom20p (Haucke *et al.*, 1995). Our results indicated that translocation of ribosome-bound Mdh1p in the presence of NAC was unaffected by the absence of either Tom20p or Tom70p. Although NAC-mediated import of nascent chains

seems to bypass these two receptor components, trypsin-sensitive proteins on the mitochondrial surface were strictly required.

The acid chain hypothesis suggests that the presequence of a precursor binds to components of the components of the TOM complex in successive steps determined by its binding affinity (Bolliger *et al.*, 1995; Dietmeier *et al.*, 1997; Schatz, 1997; Komiya *et al.*, 1998). Our results would be in agreement with a model in which NAC directs RNCs to more central parts of the TOM complex than posttranslationally imported precursors use to enter the TOM complex. The TOM channel is composed of the two essential proteins Tom22p and Tom40p and the small Tom proteins Tom5p, Tom6p, and Tom7p (Dekker *et al.*, 1998). Tom22p, Tom5p, and Tom40p have been implicated in binding to precursor proteins at later stages than Tom20p and Tom70p (Lithgow *et al.*, 1994a; Dietmeier *et al.*, 1997; Kanamori *et al.*, 1997; Rapaport *et al.*, 1997). Our results would also be in agreement with the existence of as yet unidentified proteins in the outer membrane that specifically interact with the precursor-NAC complex. However, NAC is in contact with nascent chains destined for many cellular compartments (Wiedmann *et al.*, 1994), thus a specific "NAC receptor" on the mitochondrial surface might not exist. In fact, we were unable to detect significant binding of purified NAC to trypsin-sensitive sites on the mitochondrial surface *in vitro* (our unpublished data). NAC resembles chaperones in that it unspecifically binds to unfolded proteins. Similar to many chaperone-like proteins, NAC might serve different functions in the cell. NAC positively affects translocation of RNCs into mitochondria, yet at the same time, it prevents unspecific targeting to the ER. This apparent contradiction might reflect the fundamental differences between import to these two cellular compartments. In both cases, NAC initially interacts with the nascent chain. In case of import into the ER, NAC is displaced by SRP, which is essential for specific translocation into this organelle. Specificity of mitochondrial targeting is achieved by properties that reside in the precursor protein itself. We favor the model that binding of NAC to mitochondrially targeted nascent chains affects the structure of the precursor, presenting it in a conformation that is efficiently recognized by the mitochondrial import machinery (Figure 9).

NAC probably acts on nascent chains only in the context of the ribosome (Wiedmann *et al.*, 1994). Released chains might therefore require other cytosolic factors. In our assay, import of released chains was more efficient than import of ribosome-attached nascent chains (our unpublished data). However, the import of a complex mixture of full-length mitochondrial precursor proteins (Dubaquie *et al.*, 1998) into mitochondria is significantly reduced when the translation is performed in the absence of functional NAC (our unpublished data).

Purification of NAC

We purified NAC from yeast cytosol ~900-fold. In contrast, the total import stimulatory activity was purified by a factor of 74 (Table 1).

This difference reflects a combination of at least two effects: 1) NAC might be inactivated during purification. Because immunodepletion showed that NAC constitutes a major import stimulatory activity in the salt wash fluid (Figure

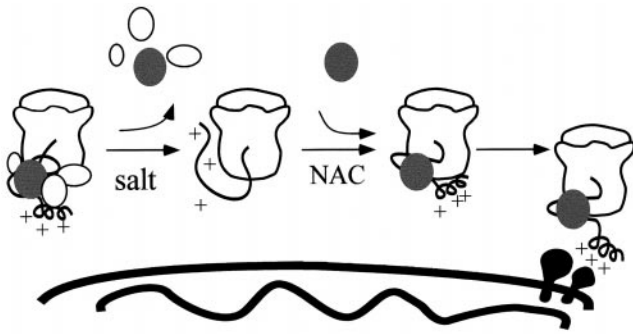


Figure 9. Proposed role of NAC in mitochondrial protein import. From left to right: untreated RNCs contain several salt-extractable proteins, including NAC (black ellipse), which keep the nascent chain import competent (depicted by the helical conformation of the positively charged presequence). Salt washing removes these proteins and exposes the nascent chain to purified MPP and lowers its import competence. Readdition of purified NAC does not shield the nascent chain against purified MPP but restores import competence of the nascent chain by presenting it in a conformation recognized by mitochondrial surface receptors.

3), loss of activity during fractionation of the salt wash fluid without concomitant loss of NAC protein suggests that NAC was either partially inactivated, or that NAC is part of a larger complex that was coimmunodepleted by the immunoserum against α NAC but was disrupted during the purification. 2) Several proteins might contribute to the total activity independently. This possibility is likely as the immunodepletion experiment showed that 20% of the stimulatory activity in the ribosomal salt wash fluid is not caused by NAC (Figure 3). This is furthermore in good agreement with the fact that NAC is not essential (Parthun *et al.*, 1992; Shi *et al.*, 1995; Reimann *et al.*, 1999).

Co- versus Post-translational Import

The question whether mitochondrial protein import *in vivo* occurs co- or post-translationally is open. Under normal growth conditions fully synthesized, but unprocessed precursor proteins are essentially not detected, suggesting that they are translocated into mitochondria either very soon after synthesis or co-translationally (Fujiki and Verner, 1993). When translation is slowed by addition of cycloheximide, yeast mitochondria are covered with ribosomes (Kellems *et al.*, 1974; Kellems *et al.*, 1975), suggesting that the ribosome-bound precursors accumulate on the surface of mitochondria. On the other hand, import can occur post-translationally *in vivo*: mitochondrial precursor proteins, accumulated *in vivo* by treatment of yeast cells with the uncoupler carbonyl cyanide *m*-chlorophenyl-hydrazone, can be chased subsequently into mitochondria by removal of carbonyl cyanide *m*-chlorophenyl-hydrazone (Reid and Schatz, 1982). Chimeric precursor proteins with the potential to form a stably folded C-terminal domain can accumulate *in vivo* as two-membrane-spanning intermediates with a folded domain outside mitochondria (Wienhues *et al.*, 1991; Schulke *et al.*, 1997). This indicates that protein synthesis is at least not coupled tightly enough with import to prevent folding of the C-terminal domain. It was proposed that

co-translational import might be involved in sorting of proteins that are found in dual locations in both mitochondria and the cytosol (Stein *et al.*, 1994; Nobumoto *et al.*, 1998).

For the first time, we show that ribosome-attached nascent chains can initiate import into mitochondria in a homologous *in vitro* system. This import system now enables us to combine mutant mitochondria (e.g., in the Tom proteins) with cytosolic extracts from mutant strains (e.g., NAC and cytosolic hsp70s). We hope to develop a more detailed understanding of the early steps of mitochondrial protein import that can facilitate *in vivo* investigation of the process.

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