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The Intermembrane Space Domain of Mitochondrial Tom22 Functions as a *trans* Binding Site for Preproteins with N-Terminal Targeting Sequences

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Mitochondrial protein import is thought to involve the sequential interaction of preproteins with binding sites on *cis* and *trans* sides of the membranes. For translocation across the outer membrane, preproteins first interact with the cytosolic domains of import receptors (*cis*) and then are translocated through a general import pore, in a process proposed to involve binding to a *trans* site on the intermembrane space (IMS) side. Controversial results have been reported for the role of the IMS domain of the essential outer membrane protein Tom22 in formation of the *trans* site. We show with different mutant mitochondria that a lack of the IMS domain only moderately reduces the direct import of preproteins with N-terminal targeting sequences. The dependence of import on the IMS domain of Tom22 is significantly enhanced by removing the cytosolic domains of import receptors or by performing import in two steps, i.e., accumulation of a preprotein at the outer membrane in the absence of a membrane potential ($\Delta\psi$) and subsequent import after reestablishment of a $\Delta\psi$. After the removal of cytosolic receptor domains, two-step import of a cleavable preprotein strictly requires the IMS domain. In contrast, preproteins with internal targeting information do not depend on the IMS domain of Tom22. We conclude that the negatively charged IMS domain of Tom22 functions as a *trans* binding site for preproteins with N-terminal targeting sequences, in agreement with the acid chain hypothesis of mitochondrial protein import.

More than 98% of mitochondrial proteins are synthesized as precursors on cytosolic polysomes, are targeted to the mitochondrial surface, and are translocated into or across the mitochondrial outer and inner membranes (20, 29, 38). The majority of preproteins contain positively charged N-terminal signal sequences, termed presequences, that are removed after import (cleavable preproteins). Other preproteins contain internal targeting sequences that remain part of the mature protein. In the past, numerous components of the mitochondrial protein import machinery have been identified, yet the molecular mechanism of translocation of preproteins is poorly understood. A widely discussed model is that protein import is mediated by the sequential interaction of preproteins with binding sites on the *cis* and *trans* sides of the membranes (3, 16, 25, 31, 32, 40). A molecular identification of *cis* and *trans* binding sites is thus of crucial importance for an understanding of the translocation process.

A multisubunit protein complex, the translocase of the outer mitochondrial membrane (Tom), contains the receptor proteins Tom70-Tom37 and Tom20-Tom22 that recognize preproteins at the cytosolic (*cis*) side of the membrane (for reviews, see references 20, 29, 37, and 38). The preproteins are translocated through a general import pore and are thought to interact with a *trans* site located on the intermembrane space (IMS) side of the outer membrane; a candidate is the IMS domain of Tom22 (3, 16, 24, 25). Subsequently, the preproteins are transferred to the translocase of the inner membrane (Tim) (28, 35, 38). A *cis* binding site on the inner membrane may be provided by the IMS domain of Tim23 (2, 6, 8). Entrance into the Tim machinery requires a membrane potential ($\Delta\psi$) across the inner membrane. The matrix heat shock protein Hsp70 binds the precursor polypeptide chain at the *trans* side of the inner membrane and, by a reversible interaction with the Tim machinery, pulls the preprotein across the membrane (17, 39, 44).

Tom22 is a central component of the outer membrane translocase. The highly negatively charged N-terminal domain on the cytosolic side seems to bind the positively charged presequences (16, 18, 21). A single transmembrane segment anchors Tom22 in the outer membrane. A small C-terminal domain (33 amino acid residues in the yeast *Saccharomyces cerevisiae*) is enriched in negative residues and has been proposed to function as a *trans* binding site for presequences (3, 16, 21, 24). The characterization of *S. cerevisiae* mutant mitochondria lacking the C-terminal IMS domain of Tom22 yielded controversial results. Nakai et al. (26) reported that mitochondrial protein import was not significantly impaired. In contrast, Bolliger et al. (3) found a strong reduction of protein import with the mutant mitochondria and performed studies of binding of synthetic targeting peptides to the purified C-terminal domain. They concluded that the IMS domain of Tom22 acts as a binding site for preproteins. Deletion of the IMS domain of *Neurospora crassa* Tom22 caused a moderate and variable reduction in import of some preproteins. By analyzing the binding of preproteins to purified outer membrane vesicles, however, Court et al. (4) concluded that the IMS domain of Tom22 was not required for formation of a *trans* site for preprotein binding.

The relevance and function of the IMS domain of Tom22 are thus unclear. Possible explanations for the opposing results are as follows. (i) The C-terminal deletions of Tom22 were not

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

Strain	Genotype	Reference
OL551	*his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1 ura3-52/ura3-52 trp1-Δ63/trp1-Δ63 TOM22/tom22::HIS3*	16
OL551-AH47	*his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1 ura3-52/ura3-52 trp1-Δ63/trp1-Δ63 TOM22/tom22::HIS3* + pRS416(*URA3*)-*TOM22*	This study
OL222	*his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 TOM22*	This study
OL200-AH47	*his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 tom22::HIS3* + pRS416(*URA3*)-*TOM22*	This study
OL200-AH46	*his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 tom22::HIS3* + pRS414(*TRP1*)-*TOM22*	This study
OL200-AH48	*his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 tom22::HIS3* + pRS414(*TRP1*)-*tom22-3*	This study
OL200-AH49	*his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 tom22::HIS3* + pRS414(*TRP1*)-*tom22-2*	This study
OL200-AH50	*his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 tom22::HIS3* + pRS414(*TRP1*)-*tom22-1*	This study

made at exactly the same position of the protein, causing truncated forms with distinct C-terminal residues (even with different charges) that may be functionally different. (ii) The assays for *trans*-site binding used isolated outer membrane vesicles (4) or the purified IMS domain of Tom22 or a preprotein stably arrested in mitochondria (3) but did not measure the productive interaction of a preprotein with the postulated *trans* site, i.e., reversible accumulation at the *trans* site followed by chase of the preprotein into mitochondria.

For this report, we attempted to clarify the role of the IMS domain of Tom22 and to address the importance of *trans* sites at the outer membrane. (i) Three distinct C-terminal deletions of *S. cerevisiae* Tom22 were constructed. They were found to be fully comparable, excluding the possibility that the reported differences were caused by distinct C termini of the truncated Tom22. (ii) We employed a functional assay for productive *trans*-site binding of preproteins with intact mitochondria. Our results strongly suggest that the IMS domain of Tom22 represents the functional *trans* site for preproteins with N-terminal targeting sequences. Binding to this *trans* site, however, is not crucial for direct import of preproteins, explaining the previous controversial conclusions. (iii) We also studied preproteins with internal targeting information and found that their import was independent of the IMS domain of Tom22.

MATERIALS AND METHODS

***S. cerevisiae* strains and growth media.** The *S. cerevisiae* strains used in this study are listed in Table 1. For isolation of mitochondria, strains were grown in yeast extract-peptone medium containing 3% glycerol (YPG medium). Selection for plasmids was done by growing the yeast on synthetic drop-out media containing 2% glucose.

Generation of plasmids and mutant yeast strains. A yeast genomic library in vector Yep13 was screened by using a digoxigenin-labeled DNA probe made from the plasmid pG-1(*TOM22*) (16). One clone containing the complete *TOM22* gene was isolated (clone AH36). After restriction analysis of clone AH36, a 3-kb *Eco*RI fragment of this plasmid was subcloned into vector pRS416 (generating plasmid AH47) and vector pRS414 (generating plasmid AH46). This 3-kb *Eco*RI fragment contained the *TOM22* open reading frame (456 nucleotides) with 1.5 kb of the 5′ and 1 kb of the 3′ noncoding sequences. Apart from the *TOM22* gene, the 3-kb *Eco*RI fragment did not contain any other complete open reading frame (22).

To introduce different mutations into the *TOM22* gene on plasmid AH46, a method described by Gatlin et al. (10) was used. The following mutated primers were designed: primer 1, 5′-GAGAGACGTCTTA**TTA**ATTGGCTCTCATGG CAAGTATAGATAAGGATAGTGGCAC-3′; primer 2, 5′-GAGAGACGTCT TA**TTA**TTGTTCGGCAAGTATAGATAAGGATAGTGGCAC-3′; primer 3, 5′-GAGAGACGTCTTA**TTA**GGCAAGTATAGATAAGGATAGTGGCAC-3′; and primer 4, 5′-GAGAGACGTCCAGCTAATCGAAATGGAAAAGACA TTTG-3′ (stop codons are shown in boldface, and *Aat*II restriction sites are underlined). Three PCRs were performed with plasmid AH46 as template DNA. The generated 8-kb products were digested with *Aat*II and *Dpn*I and ligated, yielding the plasmids AH48 (primers 1 and 4; *tom22-3*), AH49 (primers 2 and 4; *tom22-2*), and AH50 (primers 3 and 4; *tom22-1*). All three plasmids were sequenced and shown to contain the desired mutations (see Fig. 1A). Yeast strain OL551 was transformed with plasmid AH47. The resulting strain, OL551-AH47, was sporulated, and the haploid strain OL200-AH47 was selected on medium lacking histidine and uracil. Plasmids AH46 (wild type), AH48 (*tom22-3*), AH49 (*tom22-2*), and AH50 (*tom22-1*) were shuffled into strain OL200-AH47 by growing the transformants on plates containing 5-fluoroorotic acid, thus generating strains OL200-AH46 (wild type), OL200-AH48 (*tom22-3*), OL200-AH49 (*tom22-2*), and OL200-AH50 (*tom22-1*). The haploid strain OL222 was generated by sporulating OL551.

Isolation of mitochondria and in vitro import of preproteins. Yeast mitochondria were isolated as described previously (5, 12). Preproteins were synthesized in rabbit reticulocyte lysates in the presence of [^{35}S]methionine-[^{35}S]cysteine (43). Import reactions were performed in bovine serum albumin (BSA)-containing buffer (250 mM sucrose, 3% [wt/vol] fatty-acid-free BSA, 80 mM KCl, 5 mM $MgCl_2$, and 10 mM MOPS [morpholinepropanesulfonic acid]-KOH [pH 7.2]) including 2 mM ATP, 2 mM NADH, and 100 μg of mitochondrial protein/ml. Import reactions were performed at 25°C for the times indicated below. Mitochondria were reisolated by centrifugation at 16,000 × *g* for 10 min at 2°C. Samples were treated with proteinase K (100 to 200 μg/ml) for 15 min at 0°C. The proteinase K was inactivated by addition of 1 mM phenylmethylsulfonyl fluoride and incubation for 10 min at 0°C. After a washing step with SEM (250 mM sucrose, 1 mM EDTA, 10 mM MOPS-KOH [pH 7.2]), pelleted mitochondria were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Dissipation of the $\Delta\psi$ was accomplished by the addition of a mixture of valinomycin, oligomycin, and antimycin A (final concentrations of 0.5, 20, and 8 μM, respectively) (1).

The surface domains of mitochondrial receptors were removed by the treatment of mitochondria with 20 μg of trypsin per ml for 20 min at 0°C prior to the import reaction. The protease was inactivated by the addition of a 30-fold weight excess of soybean trypsin inhibitor and a 10-min incubation at 0°C.

For reduction of the $\Delta\psi$ across the inner membrane by partial uncoupling of mitochondria, the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Sigma; from a 100-fold-concentrated stock solution in ethanol) was included in the import reaction mixtures (9, 23).

The two-step import of Su9-dihydrofolate reductase (Su9-DHFR) was performed essentially as described previously (15). Mitochondria (100 μg of protein) were incubated in 300 μl of binding buffer (3% [wt/vol] BSA, 250 mM sucrose, 5 mM $MgCl_2$, 5 mM sodium malate, 2 mM ATP, 1 μM valinomycin, 20 mM KP_i, 10 mM MOPS-KOH [pH 7.2]) for 3 min at 0°C. A 1/20 volume of reticulocyte lysate with ^{35}S-labeled Su9-DHFR or urea-denatured precursor was added, and the incubation was continued for 5 min. After the samples were divided into three aliquots, the mitochondria were reisolated and resuspended in a corresponding volume of binding buffer or chase buffer (3% [wt/vol] BSA, 250 mM sucrose, 5 mM $MgCl_2$, 5 mM sodium malate, 2 mM ATP, 1 μM valinomycin, 20 mM NaP_i, 10 mM MOPS-NaOH [pH 7.2] containing sodium instead of potassium to generate a potassium diffusion potential). After incubation at 25°C, import was stopped by the addition of KCl (20 mM final concentration) and rapid cooling to 0°C.

The two-step import of the ADP/ATP carrier was performed as described for Su9-DHFR. After the chase, the samples either were directly treated with proteinase K (100 μg/ml) or were first swollen in EM buffer (1 mM EDTA, 10 mM MOPS-KOH [pH 7.2]) and then treated with proteinase K.

Miscellaneous methods. Standard techniques were used for manipulation of *Escherichia coli* and *S. cerevisiae* DNA, SDS-PAGE, immunodecoration, and storage phosphor imaging technology (Molecular Dynamics) (1, 11).

RESULTS

Three distinct C-terminal deletions of Tom22. Three C-terminal truncations of Tom22 were constructed (Fig. 1A). Tom22-1 comprised only the cytosolic domain and the predicted hydrophobic membrane anchor, representing the truncation used by Nakai et al. (26). Tom22-2 contained two additional amino acid residues of the Tom22 sequence immediately following the membrane anchor, including a negatively charged one. Tom22-3 contained four additional amino acid residues compared to Tom22-1, including a positively charged residue, sim-

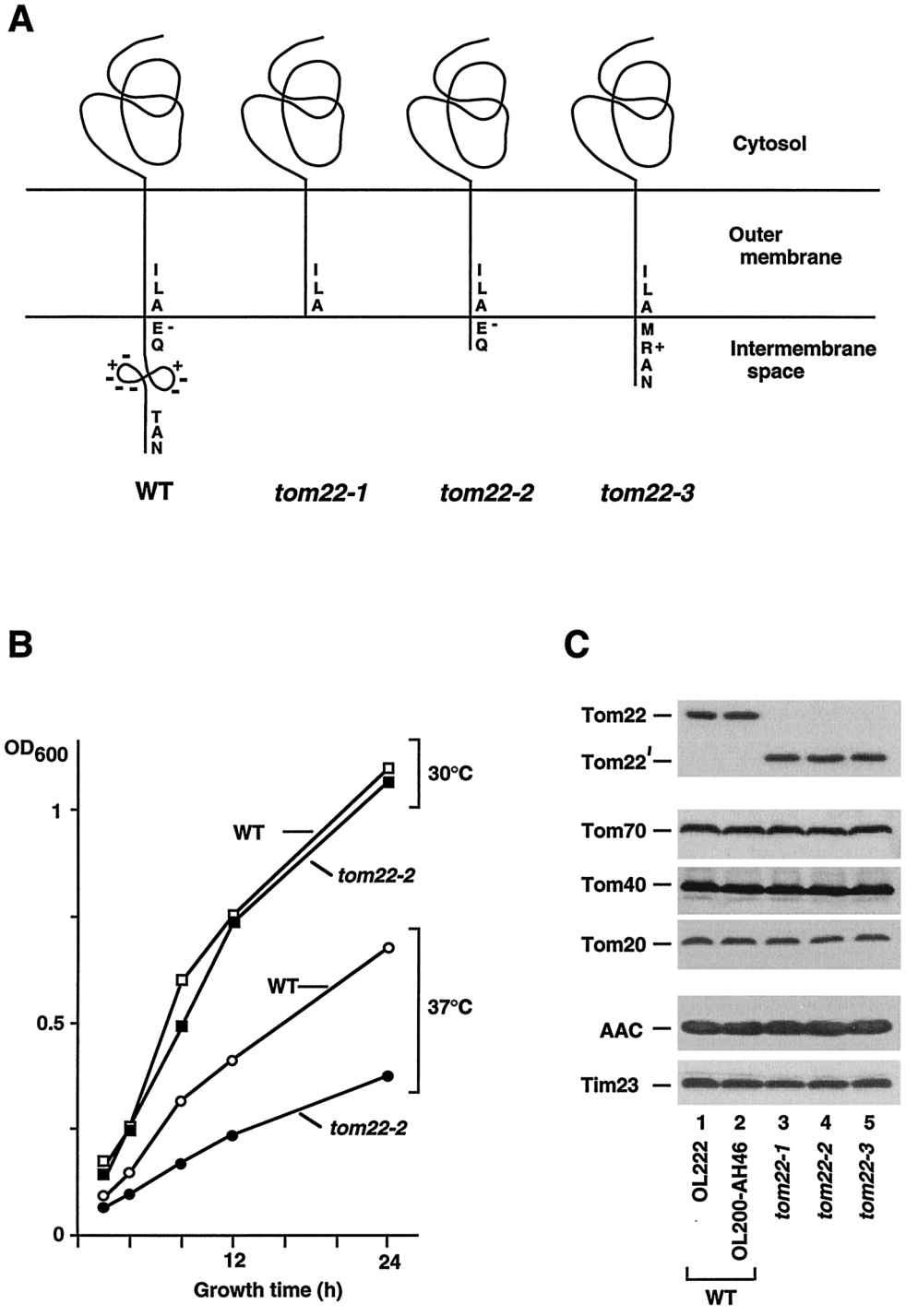

FIG. 1. Three C-terminal truncations of mitochondrial Tom22. (A) Schematic picture of wild-type (WT) Tom22 and the truncated forms of the protein. The predicted single membrane anchor of Tom22 ends with amino acid residues ILA. The C-terminal IMS domain starts with residues EQ and ends with residues TAN; it consists of 33 amino acids, including 7 negatively charged residues and 2 positively charged residues. (B) Growth phenotype of the *tom22* mutant cells. Yeast cells were grown in YPG medium (containing 3% glycerol as a carbon source) at 30 and 37°C. Cell density was determined at the times indicated by measuring the optical density at 600 nm (OD_{600}). The growth rates of the three *tom22* mutants were indistinguishable. The growth curves for strains OL200-AH46 (WT) and OL200-AH49 (*tom22-2*) are shown here. (C) Total amounts of Tom70, Tom40, Tom20, Tim23, and ADP/ATP carrier (AAC) are not changed in the mitochondria from the different *tom22* mutants (isolated after growth of the cells at 30°C). Mitochondrial proteins from yeast strains OL222 (WT), OL200-AH46 (WT; yeast strain with chromosomal deletion of *TOM22*, expressing Tom22 from a centromeric vector), OL200-AH50 (*tom22-1*), OL200-AH49 (*tom22-2*), and OL200-AH48 (*tom22-3*) (50 μg of protein each) were separated by SDS-PAGE, transferred to nitrocellulose, and immunodecorated with antisera directed against Tom22, Tom70, Tom40, Tom20, Tim23, and AAC. Tom22′, truncated Tom22.

ilar to the truncation described by Bolliger et al. (3); the last two residues of Tom22-3 were identical to the last two residues of wild-type Tom22 (Fig. 1A). The truncated proteins were expressed from single-copy yeast vectors under the control of the authentic *TOM22* promoter. Expression of any of the truncated forms of Tom22 together with wild-type Tom22 in haploid or diploid cells did not lead to a detectable growth phenotype (not shown). Three yeast strains with a deletion of the chromosomal copy of *TOM22* that expressed only the truncated forms of Tom22 were constructed. At 30°C, the mutant strains grew like the wild type. Growth of the mutant strains was reduced on a nonfermentable carbon source at an elevated temperature (Fig. 1B [results are shown for *tom22-2*; the growth curves for *tom22-1* and *tom22-3* were identical]).

The mitochondrial levels of the truncated forms of Tom22 (Fig. 1C, lanes 3 to 5) were comparable to that of wild-type Tom22 expressed from the chromosomal gene (Fig. 1C, lane 1) or from the single-copy vector (Fig. 1C, lane 2). Other components of the outer membrane translocase, such as Tom70, Tom40, and Tom20, were present in equal amounts in the wild type and the three mutants (Fig. 1C). Similarly, the levels of other marker proteins, such as the inner membrane proteins ADP/ATP carrier and Tim23, were not changed (Fig. 1C).

We conclude that mutants with the three C-terminal deletions of Tom22 are indistinguishable in their growth behavior and the composition of the mitochondrial proteins. Therefore, the different growth phenotypes previously reported, a strong growth defect on fermentable and nonfermentable carbon sources (3) versus no significant growth defect (26), are not attributable to the use of distinct Tom22 constructs. Like Bolliger et al. (3), we observed a reduction of growth, although the growth defect was limited to nonfermentable carbon sources at an elevated temperature. The comparable growth of the wild-type and mutant strains at 30°C agrees with the report of Nakai et al. (26); they did not determine growth at an elevated temperature.

Deletion of the IMS domain of Tom22 moderately reduces import of preproteins with N-terminal targeting signals. Four mitochondrial preproteins were synthesized in vitro in rabbit reticulocyte lysates in the presence of [^{35}S]methionine-[^{35}S]cysteine. Two cleavable preproteins were used: the fusion protein Su9-DHFR, consisting of the presequence of *N. crassa* F_0-ATPase subunit 9 and DHFR (32), is targeted to the matrix space (Fig. 2A); F_1-ATPase subunit β (F_1β) is targeted to the matrix side of the inner membrane (Fig. 2B). The other two preproteins contained internal targeting information and were not cleavable: the ADP/ATP carrier is transported to the inner membrane (Fig. 2C), and porin is transported to the outer membrane (Fig. 2D). The preproteins were incubated with isolated mitochondria from the wild type or the *tom22-1*, *tom22-2*, and *tom22-3* mutants in the presence of a Δψ across the inner membrane (Fig. 2A to C, lanes 1 to 3, 5 to 7, 9 to 11, and 13 to 15, and D) or in the absence of a Δψ (Fig. 2A to C, lanes 4, 8, 12, and 16). Subsequently, the mitochondria were treated with protease to remove nonimported preproteins. The reisolated mitochondria were analyzed by SDS-PAGE and digital autoradiography. Figure 2A and B show that Su9-DHFR and F_1β were processed to the mature-size forms in the presence of a Δψ. The amount of protease-protected protein is taken as a measurement of mitochondrial import of the preprotein (1). With all three mutant mitochondria, import of Su9-DHFR and F_1β was moderately yet significantly reduced by ~30% compared to import into wild-type mitochondria (Fig. 2A and B, graphs). The degree of import inhibition was indistinguishable for the three mutant mitochondria. With the noncleavable preproteins ADP/ATP carrier and porin, the rates of import into wild-type and mutant mitochondria were identical (Fig. 2C and D).

We tested a broad range of import conditions, salt concentrations (40 to 200 mM KCl), mitochondrial concentrations (20 μg to 1 mg of protein/ml), and concentrations of added BSA (0 to 3%), yet we did not observe any significant influence on the import into mutant mitochondria compared to wild-type mitochondria (not shown). We conclude that the C-terminal truncations of Tom22 moderately reduce the import of preproteins with N-terminal targeting sequence but do not affect the import of preproteins with internal targeting information.

Removal of surface receptor domains, but not a reduction of the Δψ, enhances the protein import defect of mitochondria lacking the IMS domain of Tom22. If the IMS domain of Tom22 is involved in preprotein import, it should interact with preproteins after their initial recognition by the receptor domains on the mitochondrial surface but before the Δψ-dependent insertion into the inner membrane. We thus examined whether an inhibitory effect of the truncation of Tom22 was more pronounced when an import step preceding or following it was also inhibited.

First, we removed the cytosolic domains of the import receptors by a pretreatment of mitochondria with trypsin. The mitochondria are still able to import preproteins but with reduced efficiency (~15 to 30% compared to that of intact mitochondria); this residual import is termed bypass import (1, 15, 27). The bypass import of Su9-DHFR and F_1β was significantly inhibited by the truncations of Tom22 (Fig. 3A, lanes 3 to 8, and B, lanes 5 to 7). The inhibition in comparison to the import in wild-type mitochondria was ~70%. As before, no difference between the *tom22-1*, *tom22-2*, and *tom22-3* truncations could be observed, so in further experiments mainly *tom22-2* was used. Bypass import of ADP/ATP carrier and porin was not inhibited by the truncation of Tom22 (Fig. 3C). Therefore, the importance of the IMS domain of Tom22 for import of cleavable preproteins is more prominent when the surface receptor domains are removed and preproteins are directly inserted into the general import pore. Import of preproteins with internal targeting information does not require the IMS domain of Tom22.

After traversing the outer membrane, cleavable preproteins are inserted into and translocated across the inner mitochondrial membrane. Typically, the presequence first enters the inner membrane in a step driven by the Δψ (23). We tested if mitochondria lacking the C terminus of Tom22 were able to generate a Δψ like wild-type mitochondria. The Δψ was assessed by the use of the Δψ-sensitive fluorescent dye 3,3′-dipropylthiadicarbocyanine iodide [$DiSC_3(5)$] (Fig. 4A). A decrease in fluorescence indicates the generation of a Δψ (7, 9, 41, 46). *tom22-2* mitochondria were not compromised in generation of a Δψ (Fig. 4A).

We questioned whether the dependence of import on the IMS domain of Tom22 was stronger after reduction of the Δψ. Lowering of the Δψ can be achieved by a partial uncoupling of mitochondria with limiting concentrations of the protonophore CCCP. The import of preproteins into wild-type mitochondria is thereby partially inhibited (23). Su9-DHFR and F_1β show a differential dependence on Δψ; F_1β requires a higher Δψ for protein import and is therefore more strongly inhibited already at lower concentrations of CCCP (Fig. 4B) (23). Truncation of Tom22, however, did not significantly enhance the Δψ dependence of protein import, i.e., also in the presence of CCCP, the import into *tom22-2* mitochondria was reduced by ~25 to 35% compared to that in wild-type mitochondria (Fig. 4B). Thus, no evidence for a functional relationship between the IMS do-

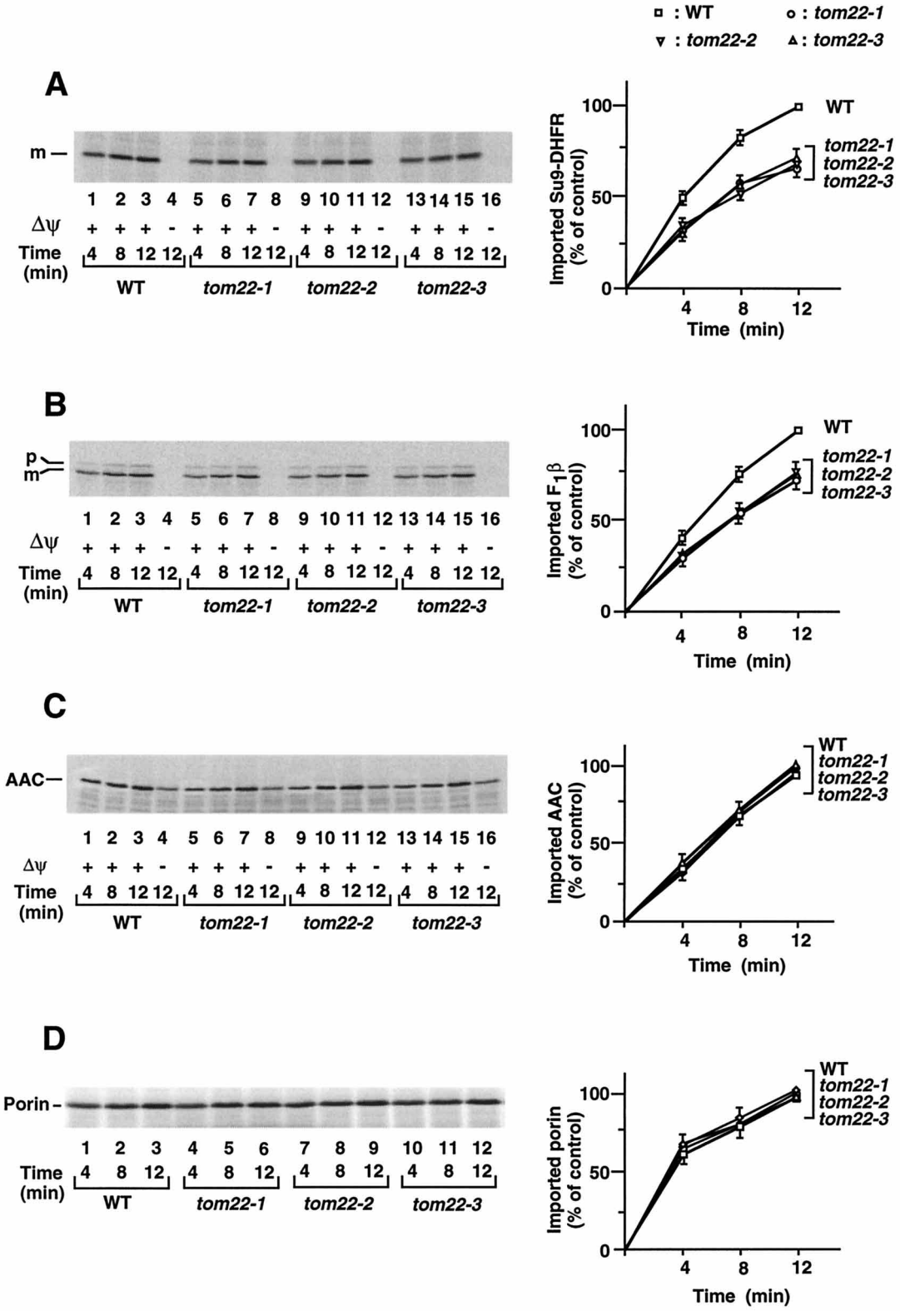

FIG. 2. Deletion of the IMS domain of Tom22 moderately reduces import of cleavable preproteins. Rabbit reticulocyte lysate with radiolabeled mitochondrial preproteins was incubated with mitochondria isolated from the wild type (WT) or *tom22* mutants (*tom22-1*, *tom22-2*, and *tom22-3*; 100 μg of mitochondrial protein/ml) for the indicated times in the presence (+) or absence (−) of a $\Delta\psi$. The preproteins used were Su9-DHFR (A), F_1-ATPase subunit β ($F_1\beta$) (B), ADP/ATP carrier (AAC) (C), and porin (D). Import was stopped by addition of 1 μM valinomycin, and the mitochondria were treated with proteinase K. After reisolation and separation by SDS-PAGE, the amounts of imported proteins were quantified by digital autoradiography. The amount of protein imported into WT mitochondria after 12 min was set to 100% (control). In the case of AAC, the amount of preprotein protected against proteinase K in the absence of a $\Delta\psi$ (at each time point) was subtracted from the amount protected in the presence of a $\Delta\psi$ to determine the amount of AAC imported into the inner membrane (1). Bars indicate the standard errors of the means (from four or five independent experiments). m, mature form; p, precursor form.

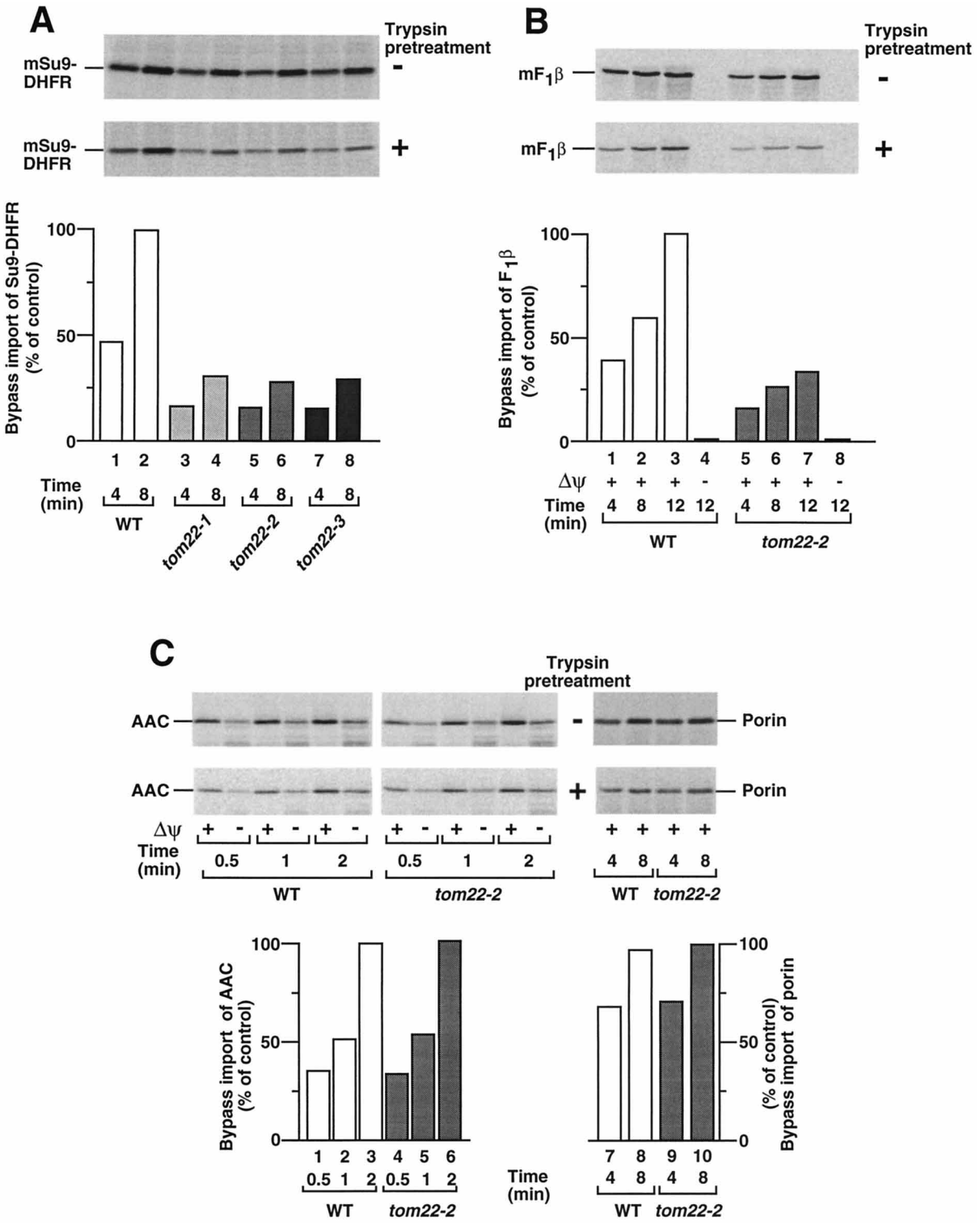

FIG. 3. The bypass import of cleavable preproteins is strongly inhibited by deletion of the IMS domain of Tom22. The receptors on the outer membrane of mitochondria were removed prior to the import reaction by a treatment with 20 μg of trypsin per ml (+) or were untreated (−). (A) Direct and bypass import of Su9-DHFR into wild-type (WT) and *tom22* mutant mitochondria was compared at the indicated times. The amount of protein imported into trypsinized mitochondria after 8 min was set to 100% (control). Quantifications are shown for the bypass import. (B) Direct and bypass import of $F_1\beta$ into WT and *tom22-2* mitochondria. The amount of protein imported into trypsinized mitochondria after 12 min was set to 100% (control). mSu9-DHFR and $mF_1\beta$, mature-form Su9-DHFR and $F_1\beta$, respectively. (C) Direct and bypass import of proteins with internal targeting sequences is not impaired by a lack of the IMS domain of Tom22. ADP/ATP carrier (AAC) and porin were imported into WT and *tom22-2* mitochondria. After the times indicated, the mitochondria were incubated with proteinase K (100 μg/ml for porin and 250 μg/ml for AAC). The amounts of protein imported into trypsinized WT mitochondria after 2 min for AAC and after 8 min for porin were set to 100% (control).

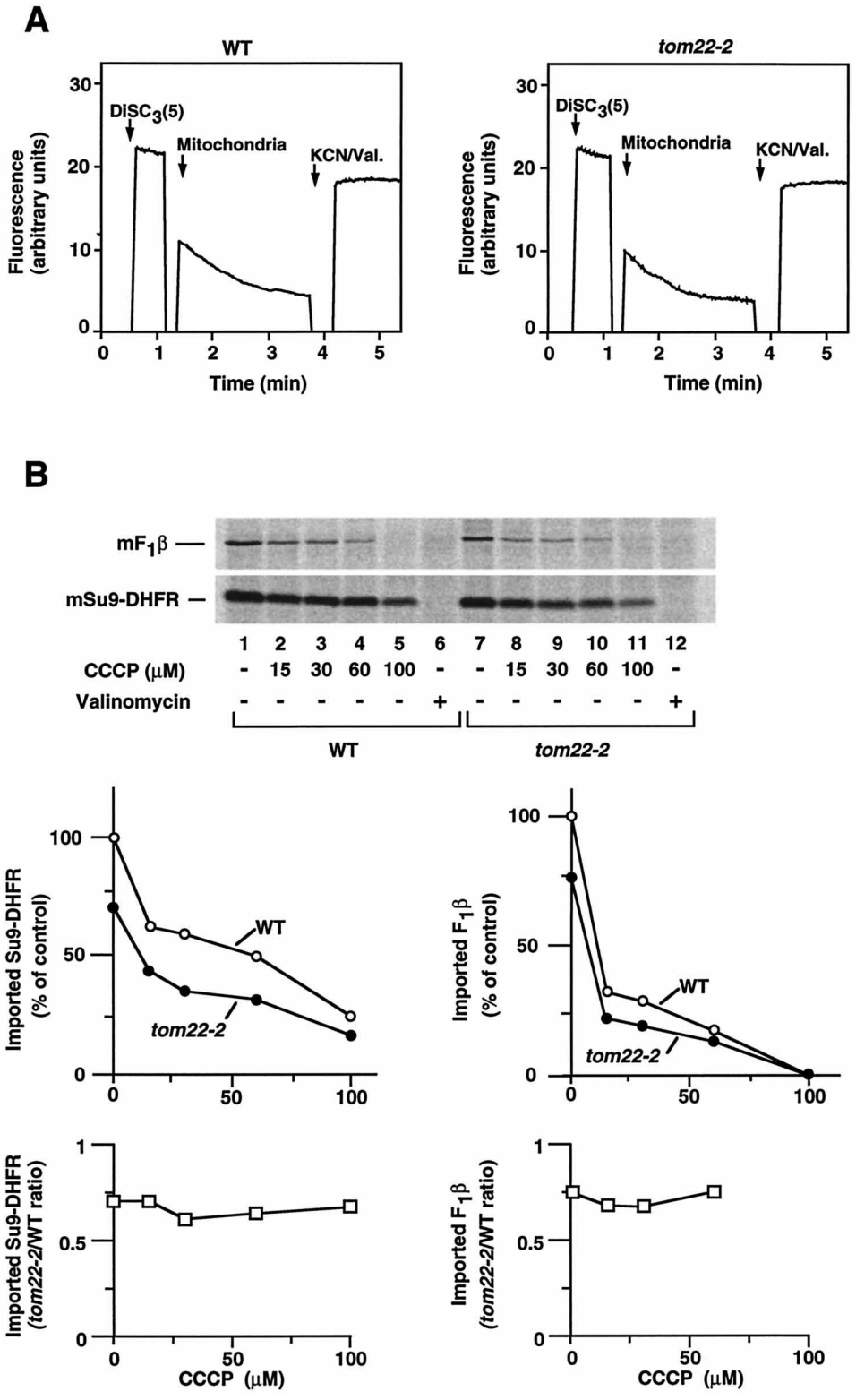

FIG. 4. Reduction of the $\Delta\psi$ does not enhance the dependence of protein import on the IMS domain of Tom22. (A) Mitochondria from the *tom22-2* mutant are able to generate a $\Delta\psi$ like that of wild-type (WT) mitochondria. The $\Delta\psi$ was assessed at 25°C by using the fluorescent dye $DiSC_3(5)$. The $\Delta\psi$ is indicated by the difference before and after the addition of potassium cyanide plus valinomycin (KCN/Val.). (B) The import of preproteins into WT and *tom22-2* mitochondria shows comparable dependence on $\Delta\psi$. Isolated mitochondria (100 μg of protein) were incubated in import buffer containing 1% BSA in the presence of 2 mM ATP and 2 mM NADH. The mitochondria were partially uncoupled by the addition of CCCP and an incubation at 25°C for 5 min prior to the import reaction. Then the ^{35}S-labeled preproteins $F_1\beta$ and Su9-DHFR were imported for 6 min, the reactions were stopped with 1 μM valinomycin (in samples 6 and 12, valinomycin was added prior to the import reaction), and a treatment with proteinase K was performed. After reisolation and separation by SDS-PAGE, the processed proteins were quantified by digital autoradiography. Import reaction mixtures without CCCP or valinomycin were used as controls (100%). The lower panel shows the ratio between the import into *tom22-2* mitochondria and that into WT mitochondria at different concentrations of CCCP. $mF_1\beta$ and mSu9-DHFR, mature-form $F_1\beta$ and Su9-DHFR, respectively.

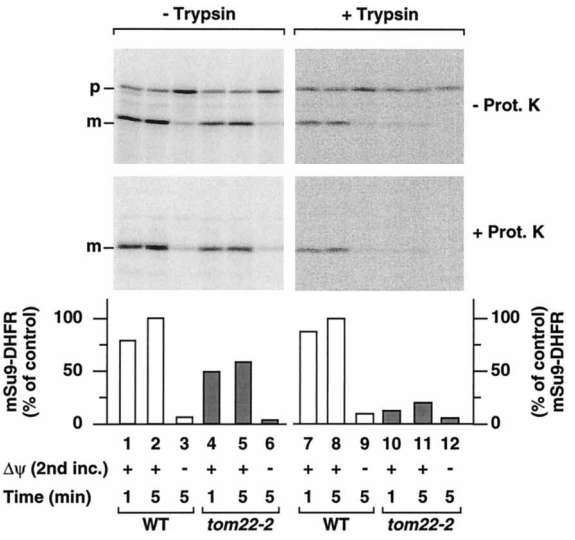

FIG. 5. Two-step import of a cleavable preprotein depends on the IMS domain of Tom22. Isolated wild-type (WT) or *tom22-2* mitochondria were pretreated with trypsin (20 μg/ml) (samples 7 to 12) or mock treated (samples 1 to 6). The fusion protein Su9-DHFR was synthesized in rabbit reticulocyte lysate in the presence of [^{35}S]methionine-[^{35}S]cysteine, precipitated with ammonium sulfate, and dissolved in 8 M urea. Urea-denatured Su9-DHFR was diluted 20-fold into binding buffer containing the reisolated mitochondria in the absence of a Δψ (valinomycin plus potassium) and incubated for 5 min at 0°C as described in Materials and Methods (15). The samples were split into three parts, and the mitochondria were reisolated. One aliquot was resuspended in the same buffer for the second incubation [− Δψ (2nd inc.)] (samples 3, 6, 9 and 12); the other two aliquots were resuspended in chase buffer (no potassium) to reestablish a Δψ [+ Δψ (2nd inc.)]. The reaction mixtures were incubated at 25°C for the times indicated, and the reactions were stopped by adding KCl to dissipate the Δψ. The samples were then split into halves. One half of each sample was treated with proteinase K (+ Prot. K). Mitochondria were reisolated and analyzed by SDS-PAGE and digital autoradiography. p and m, precursor and mature forms of Su9-DHFR. The amount of protease-protected mature-form Su9-DHFR (mSu9-DHFR) after a 5-min chase was set to 100% (control) with nontrypsinized mitochondria (columns 1 to 6) and trypsinized mitochondria (columns 7 to 12). In a parallel reaction, the direct import (one incubation of 5 min at 25°C in the presence of a Δψ) of urea-denatured Su9-DHFR into *tom22-2* mitochondria was only slightly reduced (by ~5%) compared to that into WT mitochondria (not shown); thus, the two-step import into *tom22-2* mitochondria is more strongly inhibited than the direct import.

main of Tom22 and the inner Δψ in protein import could be observed.

The IMS domain of Tom22 is crucial for productive accumulation of Su9-DHFR at the *trans* site of the outer membrane. Import of preproteins can be experimentally dissected into two steps by accumulation of preproteins at the outer membrane in the absence of a Δψ and subsequent reestablishment of a Δψ that promotes complete import of the preprotein. With this two-step import (productive binding), the investigation of import functions of the outer membrane is facilitated (14, 15, 30). We studied the two-step import of Su9-DHFR by (i) dissipation of the Δψ by the potassium ionophore valinomycin (in the presence of potassium ions in the medium) and (ii) regeneration of a Δψ by reisolation of the mitochondria and incubation in a potassium-free medium in the presence of valinomycin (15). The second step induces export of potassium ions from the matrix and generation of a potassium diffusion potential with the same orientation as the physiological proton potential (positive outside). Su9-DHFR that was accumulated at the outer membrane (Fig. 5, upper panel, lane 3) was thus efficiently processed (Fig. 5, upper panel, lanes 1 and 2) and transported to a protease-protected location (Fig. 5, lower panel, lanes 1 and 2). With *tom22-2* mitochondria, the two-step import of Su9-DHFR was significantly inhibited (Fig. 5, lanes 4 and 5, and 6A, lane 7); the inhibition was stronger than that observed for the direct import of Su9-DHFR (see the legend to Fig. 5).

The accumulation of Su9-DHFR at the outer membrane during a two-step import reaction can occur at distinct locations: at the *cis* site, i.e., the surface receptor domains, and at the putative *trans* site on the IMS side. We removed the surface receptor domains by a pretreatment of mitochondria with trypsin and then performed a two-step import (Fig. 5, lanes 7 to 12). With *tom22-2* mitochondria, the two-step import into trypsinized mitochondria was blocked (Fig. 5, lanes 10 and 11), indicating that the C terminus of Tom22 is of crucial importance for productive accumulation of preproteins at the outer membrane *trans* site.

Binding of cleavable preproteins to surface receptors occurs

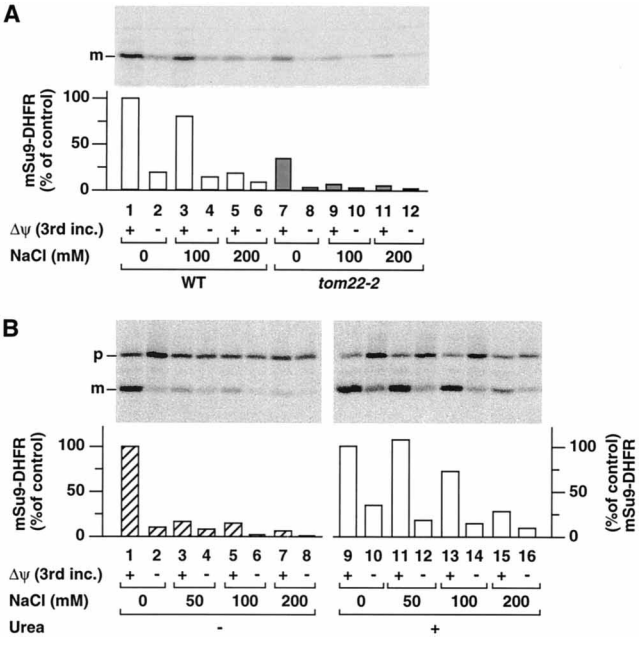

FIG. 6. The function of the IMS domain of Tom22 in productive accumulation of preproteins is salt dependent. (A) Productive binding of urea-denatured Su9-DHFR is more salt sensitive in *tom22-2* mitochondria than in wild-type (WT) mitochondria. Urea-denatured Su9-DHFR was prebound to the isolated WT and *tom22-2* mitochondria in the absence of a Δψ for 5 min at 0°C as described in Materials and Methods. The samples were split into three parts, and the mitochondria were reisolated. The aliquots were resuspended in binding buffer supplemented with 100 or 200 mM NaCl or no additional NaCl and incubated at 0°C for 15 min. Samples were split into halves, and the mitochondria were reisolated. One aliquot was again resuspended in binding buffer for the third incubation [− Δψ (3rd inc.)]; the other aliquot was resuspended in chase buffer to reestablish a Δψ [+ Δψ (3rd inc.)]. The reaction mixtures were incubated at 25°C for 5 min, the reactions were stopped, and the mixtures were treated with proteinase K to remove nonimported proteins. After reisolation of the mitochondria, proteins were separated by SDS-PAGE and analyzed by digital autoradiography. The amount of Su9-DHFR imported into WT mitochondria after a washing step without addition of NaCl was set to 100% (control). (B) Salt-resistant nonproductive binding of precursor Su9-DHFR to mitochondria. Deenergized WT mitochondria were incubated with Su9-DHFR from reticulocyte lysate (− urea) or urea-denatured Su9-DHFR for 5 min at 0°C. The samples were divided into four aliquots, and the mitochondria were reisolated. The aliquots were resuspended in binding buffer supplemented with 0 to 200 mM NaCl and incubated at 0°C for 15 min. The samples were split into halves, and the mitochondria were reisolated. One aliquot was again resuspended in binding buffer [− Δψ (3rd inc.)]; the other aliquot was resuspended in chase buffer [+ Δψ (3rd inc.)]. The reaction mixtures were incubated at 25°C for 5 min, the reactions were stopped, and the mitochondria were reisolated. p and m, precursor and mature forms of Su9-DHFR.

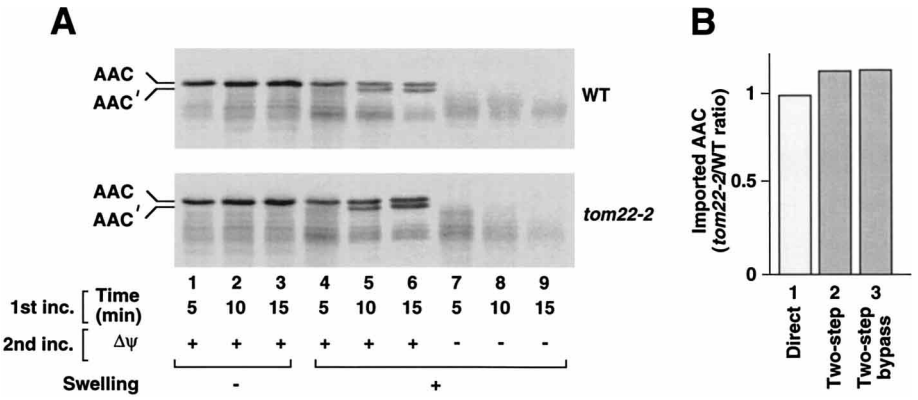

FIG. 7. Two-step import of the ADP/ATP carrier (AAC) does not require the IMS domain of Tom22. (A) The ^{35}S-labeled precursor of the AAC was accumulated at the outer membrane in the presence of valinomycin and externally added potassium ions for the times indicated. After binding, the AAC was either further imported by generation of a potassium diffusion potential (in the absence of potassium and the presence of valinomycin) ($+\Delta\psi$) or incubated in the presence of both valinomycin and potassium ions ($-\Delta\psi$). The mitochondria either were treated directly with proteinase K (100 μg/ml) or were swollen and then treated with proteinase K. AAC′, fragment of AAC correctly integrated into the inner membrane (33, 45). (B) Wild-type (WT) and *tom22-2* mitochondria were pretreated with trypsin (20 μg/ml) to test for bypass import (condition 3) or mock treated (conditions 1 and 2). After reisolation of the mitochondria, AAC was either imported directly in the presence of a $\Delta\psi$ (without accumulation of the protein at the outer membrane) or first accumulated at the outer membrane of the mitochondria for 15 min as described for panel A and then chased (two-step import). The ratio between the efficiencies of import into *tom22-2* mitochondria and WT mitochondria was calculated for direct import, two-step import, and two-step bypass import.

mainly by electrostatic interactions and is thus highly salt sensitive (13, 24), whereas interaction of preproteins with the putative *trans* site was suggested to be more salt resistant (3, 25). A major portion of the two-step import into wild-type mitochondria indeed occurred via an intermediate resistant to a treatment with 100 mM NaCl. The preprotein Su9-DHFR was accumulated at the outer membrane in the absence of a $\Delta\psi$; the mitochondria were then subjected to treatment with NaCl, followed by generation of a $\Delta\psi$ to promote complete import. The productively accumulated preprotein was resistant to a treatment with NaCl up to 100 mM (Fig. 6A, lane 3, and B, lanes 11 and 13) but not 200 mM (Fig. 6A, lane 5, and B, lane 15). Deletion of the C terminus of Tom22 inhibited the salt-resistant (100 mM monovalent salt) two-step import (Fig. 6A, lane 9). We conclude that the IMS domain of Tom22 is required for a salt-resistant productive accumulation of Su9-DHFR at the outer membrane.

Court et al. (4) reported, however, that a salt-resistant (120 mM monovalent salt) binding of Su9-DHFR to outer membrane vesicles occurred efficiently after deletion of the IMS domain of Tom22. They concluded that the C terminus of Tom22 was dispensable for *trans*-site binding. For the experiments whose results are shown in Fig. 5 and 6A, Su9-DHFR was denatured with urea to promote unfolding of the preprotein and facilitate translocation across the outer membrane; in the absence of a $\Delta\psi$, the import and unfolding motor of the Tim-matrix Hsp70 machinery cannot act on the preprotein; thus, accumulation at a *trans* site of the outer membrane was favored by denaturation of the preprotein. When the preprotein was not denatured with urea prior to import (4), most accumulated at the *cis* site of the outer membrane (25), and consequently the two-step import was salt sensitive (inhibition by 50 mM NaCl [Fig. 6B, lane 3]). Surprisingly, noncleaved preprotein accumulated at the outer membrane in a salt-resistant manner (Fig. 6B, lanes 3 to 8, p-form; in Fig. 6B, the mitochondria were not treated with proteinase K). This binding was nonproductive, as the preprotein could not be completely imported (Fig. 6B, lanes 3, 5, and 7). This result suggests that the salt-resistant binding observed by Court et al. (4) with outer membrane vesicles lacking the IMS domain of Tom22 did not represent interaction with a *trans* site but, rather, nonproductive binding to the outer membrane.

Productive accumulation of a preprotein with internal targeting information at the outer membrane does not require the IMS domain of Tom22. The precursor of the ADP/ATP carrier accumulates at the outer membrane in the absence of a $\Delta\psi$. The interaction with the outer membrane can occur at two distinct sites: a *cis* site, i.e., surface receptors, also termed stage 2; and a putative *trans* site, also termed stage 3 (stage 1 represents precursor of the ADP/ATP carrier interacting with cytosolic cofactors) (31, 32, 43). ADP/ATP carrier accumulated at stage 3 is exposed to the IMS; after rupture of the outer membrane by osmotic shock, accumulated ADP/ATP carrier remains firmly bound to the outer membrane but becomes accessible to added proteases or antibodies (1, 33, 43a).

We analyzed productive binding of the ADP/ATP carrier to the outer membrane by performing a two-step protein import: accumulation in the presence of valinomycin and external potassium and further import by generation of a potassium diffusion potential (valinomycin without external potassium). Wild-type and *tom22-2* mitochondria showed efficient two-step import, as assessed by a treatment of the mitochondria with protease after the import reaction (Fig. 7A, lanes 1 to 3). As a specific assay for correct integration of imported ADP/ATP carrier into the inner membrane, the mitochondria were swollen to open the outer membrane and treated with proteinase K, after which a characteristic fragment, AAC′, is formed only from fully imported ADP/ATP carrier (Fig. 7A, upper panel, lanes 5 and 6) (33, 45); in the absence of a $\Delta\psi$, ADP/ATP carrier is not inserted into the inner membrane and thus the fragment is not formed (Fig. 7A, upper panel, lanes 7 to 9). The efficiency of two-step import, determined by formation of the fragment AAC′, was not reduced by the truncation of Tom22 (Fig. 7A, lower panel, lanes 5 and 6, and B, column 2).

Under the conditions used for Fig. 7A, preprotein could accumulate at both a *cis* site and a *trans* site of the outer membrane. To test for selective accumulation at a *trans* site, the mitochondria were pretreated with a low concentration of trypsin to remove the surface receptor domains. The residual (bypass) two-step import into *tom22-2* mitochondria was not

reduced compared to that of wild-type mitochondria (Fig. 7B, column 3), in contrast to the observation made with Su9-DHFR (see above [Fig. 5]). We conclude that the IMS domain of Tom22 is not required for transport of the ADP/ATP carrier across the outer membrane.

DISCUSSION

We report that the IMS domain (C terminus) of mitochondrial Tom22 is of critical importance for productive accumulation of presequence-carrying preproteins at a *trans* site of the outer membrane.

Deletion of the IMS domain of Tom22 inhibited direct import of cleavable preproteins only by ~30%, indicating that in the presence of a $\Delta\psi$, a major portion of preprotein translocation across the outer membrane and transfer into the inner membrane can proceed in the absence of this part of Tom22. At first glance, this observation seems to agree with the conclusions of Nakai et al. (26) and Court et al. (4) that the IMS domain of Tom22 plays no role or only a minor role in protein import. The dependence of import on the IMS domain, however, was strongly enhanced when the cytosolic domains of the import receptors were removed, suggesting that the direct insertion of preproteins into the general import pore of the outer membrane is supported by the C terminus of Tom22.

To address the question of whether this Tom22 domain acts as a *trans* site for preproteins traversing the outer membrane, we performed a functional assay for productive accumulation of a cleavable preprotein at an outer membrane *trans* site of intact mitochondria. In a two-step import reaction, preproteins are transiently accumulated at the outer membrane before import is completed by generation of a $\Delta\psi$. The two-step import, which involves binding to both *cis* and *trans* sites, was significantly inhibited by deletion of the C terminus of Tom22. When the surface domains of import receptors were removed, two-step import involved accumulation of preproteins selectively at the *trans* site and strictly depended on the IMS domain of Tom22. This indicates that the IMS domain of Tom22 is crucial for *trans*-site binding of a presequence-carrying preprotein at the outer membrane.

Binding of cleavable preproteins to the surface receptors occurs predominantly by ionic forces and is thus highly sensitive to the addition of salt, e.g., inhibition by 50 mM monovalent salt (13, 24). Mayer et al. (25) studied preprotein interaction with a putative *trans* site of isolated outer membrane vesicles, and Bolliger et al. (3) characterized binding of a chemically synthesized presequence peptide to the purified C terminus of Tom22. Both showed that the salt resistance of interaction was higher than that observed with surface receptors. The functional assay for productive *trans*-site accumulation of a preprotein reported here revealed a similar salt resistance (resistance to 100 mM monovalent salt) that depended on the presence of the C terminus of Tom22, strongly suggesting that the IMS domain of Tom22 represented the *trans* site. Recently, however, Court et al. (4) reported that they observed salt-resistant (120 mM monovalent salt) interaction of a cleavable preprotein with isolated outer membrane vesicles independently of the presence or absence of the C terminus of Tom22. How can this controversy be explained? Court et al. (4) did not test if the presequence was translocated across the outer membrane, whereas Mayer et al. (25) analyzed only the fraction of preproteins whose presequence was translocated across the outer membrane. Accumulation at the *trans* site is done in the absence of a $\Delta\psi$ across the inner membrane, and therefore the main import-driving forces, $\Delta\psi$ and ATP-dependent action of matrix Hsp70, cannot work. We found that denaturation (unfolding) of a preprotein with urea facilitated translocation across the outer membrane and thereby strongly enhanced interaction of the bulk of the preprotein with the *trans* site. A nondenatured preprotein (Su9-DHFR), as used by Court et al. (4), indeed showed a salt-resistant binding to isolated mitochondria (up to 200 mM monovalent salt), but the binding was nonproductive, that is, the bulk of preprotein could not be further imported after generation of a $\Delta\psi$. This indicates that the salt-resistant interaction with outer membranes seen by Court et al. (4) could represent mainly nonproductive binding.

The import of noncleavable preproteins of porin and ADP/ATP carrier was not inhibited by deletion of the C terminus of Tom22, even after removal of the cytosolic receptor domains. Import of the precursor of the ADP/ATP carrier probably involves interaction with a *trans* site of the outer membrane (31–33). The results reported here demonstrate that the IMS domain of Tom22 does not function as the *trans* binding site for the ADP/ATP carrier. Bolliger et al. (3) showed that import of the noncleavable precursor of the matrix protein chaperonin 10 depended on the C terminus of Tom22. Chaperonin 10 contains a typical N-terminal targeting sequence that is not cleaved after import and thus resembles presequence-carrying preproteins (34, 36). We conclude that preproteins with internal targeting information, such as outer membrane porin and inner membrane ADP/ATP carrier, do not require the C terminus of Tom22 for import. The precursor of the ADP/ATP carrier may use a distinct *trans* site of the outer membrane that has not been identified at a molecular level. Most import steps of the ADP/ATP carrier are thus distinct from those of presequence-carrying preproteins (19): initial recognition by surface receptors (Tom70-Tom37 versus Tom20-Tom22), *trans*-site binding at the outer membrane, and transport by the inner membrane machinery (the recently identified Tim22 complex [42] versus the Tim17-Tim23-Tim44 machinery). The import pathways of ADP/ATP carrier and cleavable preproteins converge at the cytosolic domain of Tom22 and the entry into the general import pore and diverge again at the exit of the general import pore.

We conclude that the IMS domain of Tom22 is required to form the outer membrane *trans* site for preproteins with N-terminal targeting sequences. However, inactivation of the *trans* site does not fully block the direct transfer of preproteins from the general import pore of the outer membrane to the inner membrane machinery when the presence of a $\Delta\psi$ allows immediate continuation of the import process. Therefore, this *trans* site is not essential for direct import of preproteins into energized mitochondria but only enhances the translocation rate. Two explanations are conceivable: (i) direct import (with a reduced efficiency) is possible in the absence of a *trans* site, or (ii) an additional outer membrane protein participates in formation of a *trans* site and provides a weak *trans*-site activity by itself which is sufficient for direct import but not for accumulation of preproteins. In both cases, the IMS domain of Tom22 is of crucial importance when the functions of cytosolic receptor domains are impaired and when preproteins are transiently arrested at the outer membrane in the absence of a $\Delta\psi$. The C terminus of Tom22 carries a negative net charge that should facilitate interaction with the positively charged presequences (3, 16, 18, 21). The findings reported here support the acid chain hypothesis (16) that negatively charged segments of import components provide binding sites for presequence-carrying preproteins along the import pathway across outer and inner membranes and thus guide a stepwise import.

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