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Analysis of the Sorting Signals Directing NADH-Cytochrome b_5 Reductase to Two Locations within Yeast Mitochondria

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Mitochondrial NADH-cytochrome b_5 reductase (Mcr1p) is encoded by a single nuclear gene and imported into two different submitochondrial compartments: the outer membrane and the intermembrane space. We now show that the amino-terminal 47 amino acids suffice to target the Mcr1 protein to both destinations. The first 12 residues of this sequence function as a weak matrix-targeting signal; the remaining residues are mostly hydrophobic and serve as an intramitochondrial sorting signal for the outer membrane and the intermembrane space. A double point mutation within the hydrophobic region of the targeting sequence virtually abolishes the ability of the precursor to be inserted into the outer membrane but increases the efficiency of transport into the intermembrane space. Import of Mcr1p into the intermembrane space requires an electrochemical potential across the inner membrane, as well as ATP in the matrix, and is strongly impaired in mitochondria lacking Tom7p or Tim11p, two components of the translocation machineries in the outer and inner mitochondrial membranes, respectively. These results indicate that intramitochondrial sorting of the Mcr1 protein is mediated by specific interactions between the bipartite targeting sequence and components of both mitochondrial translocation systems.

Most mitochondrial proteins are synthesized in the cytoplasm and must be imported across one or both mitochondrial membranes to reach their final destinations within the organelle (18, 27, 28). Thus, precursor proteins have to contain information not only for targeting to the mitochondrion but also for sorting to the correct submitochondrial compartment (6, 33). Proteins destined for the mitochondrial matrix usually bear a positively charged amphipathic targeting sequence at their N termini (26) and reach this compartment by movement through the translocation machineries in the outer and inner mitochondrial membranes (15, 17, 25), while proteins destined for other compartments deviate from the general "matrix-targeting pathway" at different points (5, 6, 11, 31, 33, 35). The question of how intramitochondrial protein sorting occurs becomes even more difficult to answer if a protein is localized to more than one compartment within the mitochondrion (10; for a review, see reference 3). NADH-cytochrome b_5 reductase (Mcr1p) of yeast mitochondria represents the first known protein which is encoded by a single gene but transported to two different locations within the same organelle in vivo. One form, the primary translation product, is firmly anchored to the outer mitochondrial membrane, whereas about half the molecules reach the inner membrane and are then processed and released into the intermembrane space (10).

We proposed earlier (10) that this differential sorting is achieved by incomplete translocation arrest in the mitochondrial outer membrane. We suggested a model according to which (i) the extreme amino-terminal region of Mcr1p functions as a weak matrix-targeting signal whereas a downstream region functions as a sorting sequence, (ii) mutations that weaken the hydrophobic character of the sorting sequence would reduce the ability of the precursor to become anchored to the outer membrane but would increase the efficiency of transport into the intermembrane space, and (iii) specific interactions with components of both mitochondrial translocation machineries are responsible for the sorting of Mcr1p to its correct destinations.

In order to test this model, we have analyzed the in vitro import pathway of wild-type Mcr1p and several mutants and fusion proteins. The results agree with our earlier model for an unusual mechanism of protein sorting in mitochondria.

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MATERIALS AND METHODS

Construction of Mcr1 hybrid proteins and site-directed mutagenesis. (i) Mcr1(1-12)-DHFR. The first 12 amino acids of Mcr1p were fused to mouse dihydrofolate reductase (DHFR) by PCR with P.12F as the forward primer (5′-TGG CTG CAG TTA ATG TTT TCC AGA TTA TCC AGA TCT CAC TCA AAA GCA GCA ATG GTT CGA CCA TTG-3′) and P.DHFRb (5′-CCC GGC GGA TCC TTC TCG TAG ACT TCA AAC TTA-3′) as the reverse primer. The *Pst*I-*Bam*HI-digested PCR product was subcloned into pSP64 (Promega) for in vitro transcription with SP6 polymerase.

(ii) Mcr1(1-47)-DHFR. A DNA fragment encoding amino acids 1 to 47 of Mcr1p was amplified by PCR with KH.p5 (5′-TGC ACT GCA GTA AGC TTG ATG TTT TCC AGA TTA TTC C-3′) as the forward primer and P.MCR/DHFRb as the reverse primer (5′-CAA TGG TCG AAC CAT GAA TAC TTT ATT GGA-3′); a second DNA fragment, encoding amino acids 43 to 47 of Mcr1p plus the entire mouse DHFR, was amplified by PCR with P.MCR/DHFRf as the forward primer (5′-TCC AAT AAA GTA TTC ATG GTT CGA CCA TTG-3′) and P.DHFRb as the reverse primer. The DNA fragments were purified, mixed, and used as a template in a second PCR with primers KH.p5 and P.DHFRb to generate a fusion gene encoding Mcr1(1-47)-DHFRp. The *Pst*I-*Bam*HI-digested fusion gene was subcloned into pSP64 for in vitro transcription with SP6 polymerase.

(iii) AAQQ mutant of Mcr1p. Two DNA fragments were amplified by PCR with the mutagenic primers P.AAQQf (5′-TGC GGT AGC TTG TTG TAT AGC AAC TGT-3′) and P.AAQQb (5′-ACA GTT GCT ATA CAA CAA GCT ACC GCA TTC-3′), and the resulting PCR products were purified, mixed, and used as a template in a second PCR with primers KH.p5 and KH.p6 (5′-GGA ATT CAA GCT TGC TTA AAA TTT GAA AAC-3′). The *Eco*RI-*Pst*I-digested DNA fragment encoding the mutated Mcr1p was subcloned into pSP64 for in vitro transcription.

All constructs were verified by restriction analysis and double-stranded DNA sequencing.

Yeast strains. Unless stated otherwise, experiments were performed with the haploid *Saccharomyces cerevisiae* strain D273-10B (*MAT*α; ATCC 25657). The experiments shown in Fig. 6 and 7 were performed with *S. cerevisiae* strains YPH499 (*MAT***a** *ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801*) or YAH101 (*MAT*α *ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801 tom7::TRP1*) (14) and YJK9-3d**a** (*MAT***a** *ade2-101 his3 lys2-801*) or YTIM11-1 (*MAT*α *ade2-101 his3 lys2-801 tim11::KAN*).

Cell growth and isolation of mitochondria. Yeast strains were cultured on semisynthetic medium supplemented with 2% sodium lactate and 0.1% glucose. Total yeast cell lysates were prepared according to the method of Horvath and Riezman (16). Mitochondria were isolated and purified as described by Glick and Pon (8). For the experiment shown in Fig. 6A and B, mitochondria were isolated according to the method of Hartl et al. (11a).

Protein import into isolated yeast mitochondria. Precursor proteins were imported into isolated yeast mitochondria as described by Wachter et al. (38). Mitoplasts were generated after import according to the method of Glick et al. (7). To assay for membrane insertion of imported precursor proteins, the mitochondria were reisolated, washed, resuspended at 0.5 mg/ml in 100 mM Na_2CO_3 (pH 11.5), incubated on ice for 30 min, and spun at 100,000 × *g* at 4°C for 15 min. The pellet was resuspended in an equal volume of 100 mM Na_2CO_3 (pH 11.5); proteins from the pellet and supernatant were precipitated with 10% trichloroacetic acid and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), fluorography, and densitometric quantitation of the bands. To assay for protease protection of imported proteins, the mitochondria were reisolated, washed, resuspended in import buffer containing 80 μg of proteinase K/ml, and incubated on ice for 30 min; digestion was stopped by addition of 1 mM phenylmethylsulfonyl fluoride (PMSF). The mitochondria were reisolated by centrifugation at 12,000 × *g* at 4°C and resuspended in an equal volume of import buffer, and total proteins were precipitated with 10% trichloroacetic acid. Samples were analyzed by SDS-PAGE, fluorography, and densitometric quantitation of the bands.

Manipulation of ATP levels. ATP was selectively depleted from the mitochondrial matrix, from the mitochondrial exterior, or from both sides of the mitochondrial inner membrane according to the method of Wachter et al. (38).

Miscellanea. Published methods were used for SDS-PAGE, standard DNA procedures, transformation of yeast and *Escherichia coli*, in vitro transcription/translation, and immunoblotting (24, 38). Fluorograms were quantified with a computerized β-imager (Biospace Instruments, Paris, France).

RESULTS

Mcr1p is both inserted into the outer membrane and transported into the soluble intermembrane space upon import into isolated mitochondria. We first checked whether Mcr1p imported into isolated mitochondria attains a submitochondrial localization similar to that in living yeast cells. Figure 1A shows that this is indeed the case; we observed the same 34- and 32-kDa isoforms, which are found by immunoblot analysis of total yeast proteins (Fig. 1A, lane 1; compare reference 10). Generation of the 32-kDa isoform was inhibited by collapsing the electrochemical potential across the mitochondrial inner membrane (Fig. 1A, lanes 1 to 4). The 32-kDa isoform [Mrc1 (32)] was protease protected in mitochondria (Fig. 1A, lane 3) but was released from the mitochondria (Fig. 1A, lane 8) into the supernatant (data not shown) upon rupture of the outer membrane, indicating that it was a soluble protein of the intermembrane space. In contrast, the 34-kDa isoform [Mrc1 (34)] appeared to be exposed on the mitochondrial surface, as it was readily digested in intact mitochondria or mitoplasts by proteinase K (Fig. 1A, lanes 1, 3, and 9). Mcr1(34) was firmly anchored in the mitochondrial outer membrane upon import, as it was resistant to extraction by carbonate at pH 11.5 in mitochondria or mitoplasts (Fig. 1A, lanes 5 and 7). In contrast, Mcr1(32) was completely released under these conditions (Fig. 1A, lane 6). Import of the Mcr1p precursor into the soluble intermembrane space was less efficient in vitro than in vivo, consistent with our earlier findings (10). As import of both Mcr1p isoforms was strongly inhibited by pretreating the mitochondria with trypsin prior to import (Fig. 1B), it appears to require cytosolically exposed receptor proteins.

Only native, but not urea-denatured, Mcr1p requires matrix ATP for import into the intermembrane space. Protein transport across the mitochondrial inner membrane requires ATP in the matrix, reflecting the action of mitochondrial hsp70 (36). In order to test whether mhsp70 has a role in the import of Mcr1p into the intermembrane space, we analyzed the energy requirements of the import reaction. The native precursors of

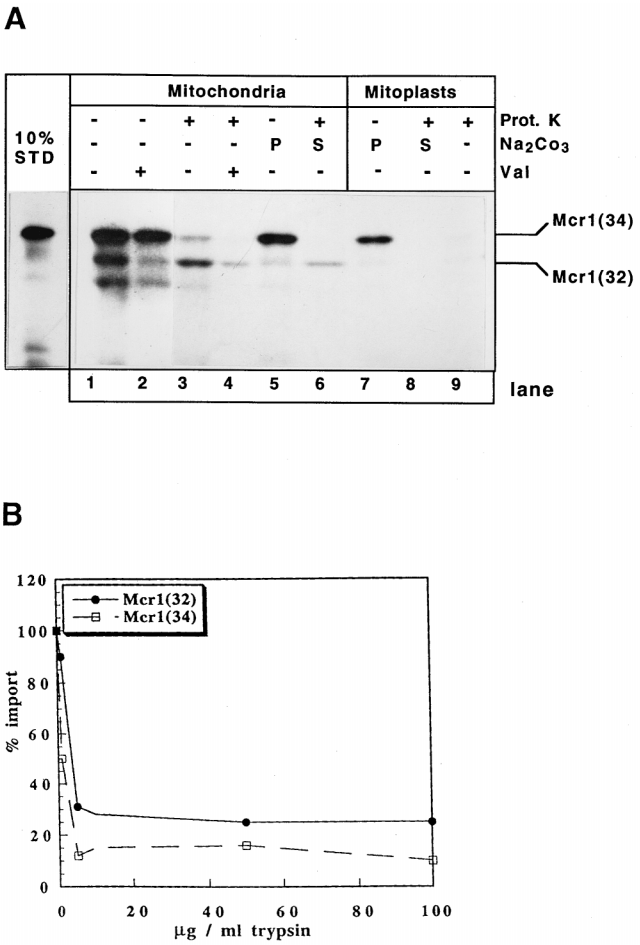

FIG. 1. Mcr1p is imported into the outer mitochondrial membrane and into the soluble intermembrane space in vitro. (A) Import of Mcr1p into isolated mitochondria. The Mcr1p precursor was synthesized in vitro and incubated with fully energized yeast mitochondria for 15 min at 25°C in the presence or absence of the K^+ ionophore valinomycin (Val), which dissipates the electrochemical potential across the inner membrane. Mitochondria were reisolated, resuspended in import buffer, and divided into aliquots. Samples were either left untreated (− Prot. K) or treated with proteinase K (+ Prot. K) for 30 min on ice. Digestion was stopped by adding 1 mM PMSF. Where indicated, mitochondria were then converted to mitoplasts to selectively rupture the outer mitochondrial membrane. The mitochondria or mitoplasts were reisolated, and the pellet was resuspended in import buffer (− Na_2CO_3) or 100 mM Na_2CO_3 (Na_2CO_3, P or S). Carbonate pellet (P) and supernatant (S) were separated as described in Materials and Methods. All samples were finally precipitated with 10% trichloroacetic acid and analyzed by SDS-PAGE and fluorography. 10% STD, 10% of the amount of precursor added to each assay. Mcr1(34), full-length 34-kDa Mcr1p; Mcr1(32), processed 32-kDa intermembrane space isoform. All lanes are from the same autoradiogram. (B) Import of Mcr1p into mitochondria pretreated with trypsin. Mitochondria were treated with 0, 1, 5, 10, 50, or 100 μg of trypsin/ml for 30 min on ice prior to import. Trypsin was inactivated by addition of soybean trypsin inhibitor to 1 mg/ml. The mitochondria were reisolated, washed, and resuspended in import buffer containing 2 mM ATP and 2 mM NADH. The in vitro-synthesized Mcr1p precursor was allowed to be imported into mitochondria as described in the legend to panel A. Insertion of Mcr1p [Mrc1(34)] into the outer mitochondrial membrane was assayed by measuring the amount of carbonate-inextractable full-length Mcr1p. Import of Mcr1p into the intermembrane space was assayed by quantifying the amount of protease-protected Mcr1(32). The amount of precursor imported into the outer membrane or the intermembrane space of untreated mitochondria was taken as 100%. This value corresponded to 8.2 and 3.4%, respectively, of the added Mcr1p precursor.

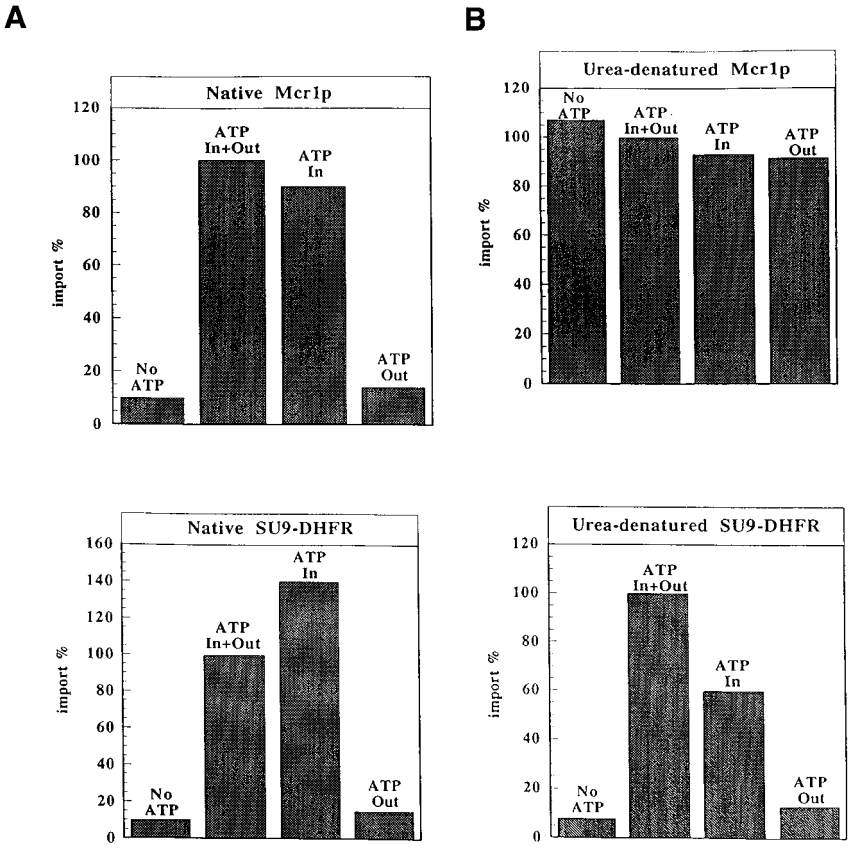

FIG. 2. Import of native, but not urea-denatured, Mcr1p into the intermembrane space requires matrix ATP. (A) ATP requirements for import of native Mcr1p and pSU9-DHFR into mitochondria. The precursors of Mcr1p and pSU9-DHFR were synthesized in vitro, and the translation mixture was subsequently depleted of ATP. The pretreated radiolabeled precursor proteins were then added to mitochondria under conditions under which ATP was present on both sides of the inner membrane (ATP In + Out), in the matrix only (ATP In), outside the inner membrane only (ATP Out), or in neither location (No ATP). After import for 8 min at 30°C, the mitochondria were reisolated, washed, and treated with proteinase K. Following addition of 1 mM PMSF, the samples were precipitated with trichloroacetic acid and analyzed by SDS-PAGE, fluorography, and quantitation of the bands. Import %, percentage of precursor imported into mitochondria that have been selectively depleted of ATP; the amount of mature SU9-DHFR or Mcr1(32) imported into fully energized mitochondria (ATP In + Out) was taken as 100%. This value corresponds to 15 or 3.6%, respectively, of the added SU9-DHFR or Mcr1p precursor. (B) ATP requirements for import of urea-denatured Mcr1p and pSU9-DHFR into mitochondria. The experiment was carried out as described in the legend to panel A, except that the precursor proteins were denatured for 5 min in 8 M urea prior to import and were diluted 20-fold into import buffer containing mitochondria that had been pretreated as described for panel A. The amount of mature SU9-DHFR or Mcr1(32) imported into fully energized mitochondria (ATP In + Out) was taken as 100%. This value corresponds to 21 or 3.8%, respectively, of the added SU9-DHFR or Mcr1p precursor.

Mcr1p (Fig. 2A, upper panel) or of SU9-DHFR (a fusion protein targeted to the mitochondrial matrix) (Fig. 2A, lower panel) were incubated with mitochondria under conditions (38) in which ATP was present in the matrix, outside the mitochondrial inner membrane, on both sides of the inner membrane, or on neither side. Both precursors were efficiently imported into the mitochondrial interior (the intermembrane space or the matrix) only if ATP was present in the mitochondrial matrix (Fig. 2A). Depletion of ATP outside the mitochondrial inner membrane had no significant effect on the import of either of these precursors (Fig. 2A). By contrast, insertion of Mcr1p into the outer mitochondrial membrane did not require ATP (data not shown). If the Mcr1p precursor was denatured in 8 M urea before being added to mitochondria, its import into the intermembrane space was ATP independent (Fig. 2B, upper panel), whereas denaturation of SU9-DHFR did not bypass the requirement for matrix ATP for import into the mitochondrial matrix (Fig. 2B, lower panel) (38). Thus, urea denaturation enables the Mcr1p precursor to bypass the matrix ATP requirement for import into the intermembrane space.

The energy requirements for import of Mcr1p into the intermembrane space closely resemble those of cytochrome b_2, another intermembrane space protein (9, 32, 36). Mcr1p is an isoform of NADH-cytochrome b_5 reductase with particularly strong homology to this family in the presumed catalytic regions which are implicated in the binding of NADH and flavin adenine dinucleotide. The requirement for matrix ATP may thus reflect the ATP requirement of mhsp70 (36), which must transiently unfold the tightly folded catalytic domain of Mcr1p during translocation across the mitochondrial outer membrane.

The information for targeting and sorting of Mcr1p to its final destinations resides in the amino-terminal 47 amino acids. We initially hypothesized that the information for intracellular targeting of Mcr1p resides in the amino-terminal 50 amino acids (10). To test this prediction, we fused mouse DHFR to the first 12 residues of Mcr1p (corresponding to the putative matrix-targeting sequence of Mcr1p) or to the first 47 amino acids of Mcr1p (corresponding to the putative matrix-targeting sequence plus the hydrophobic segment and the cleavage site for inner membrane protease I [29]) (Fig. 3A). When the urea-denatured Mcr1(1-12)-DHFR fusion protein was added to en-

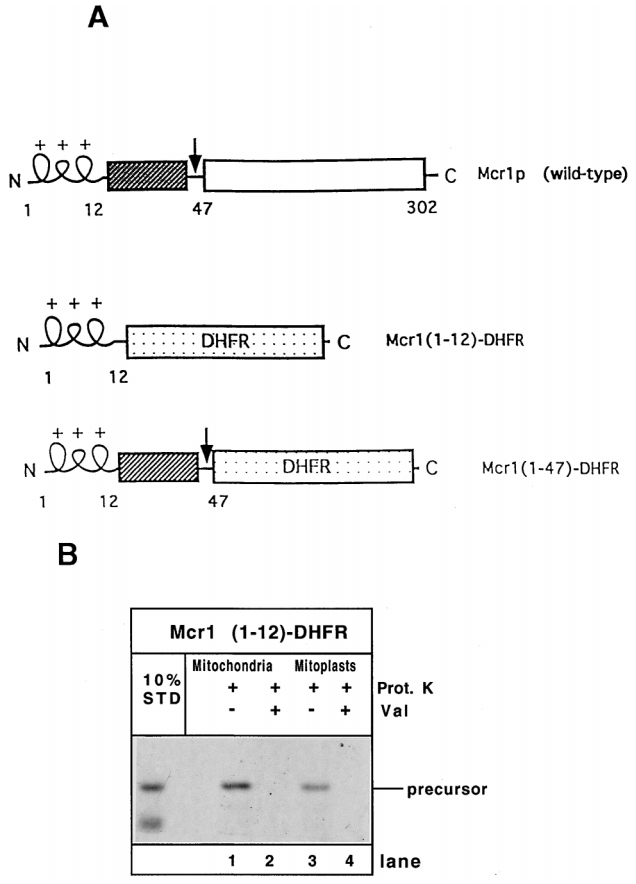

FIG. 3. The complete information for targeting and sorting of Mcr1p to its authentic locations is contained within the first 47 amino acids. (A) Schematic representation of authentic Mcr1p and two fusion proteins with DHFR. The putative matrix-targeting sequence (amino acids 1 to 12) of Mcr1p is depicted as a positively charged (plus signs) helix, the hydrophobic segment as a hatched box, mouse DHFR as a stippled rectangle, the mature part of Mcr1p as an open rectangle, and the cleavage site for inner membrane protease I by an arrow. Numbers correspond to amino acids of the Mcr1p precursor. (B) The first 12 amino acids of the Mcr1p precursor are sufficient to direct attached DHFR into the mitochondrial matrix. Mcr1(1-12)-DHFR was synthesized in vitro, denatured for 5 min in 8 M urea, and added to fully energized isolated yeast mitochondria for 10 min at 30°C in the presence (+ Val) or absence (− Val) of the K^+ ionophore valinomycin. Mitochondria were reisolated, resuspended in import buffer, and divided into aliquots. Samples were either left untreated (− Prot. K) or treated with proteinase K (+ Prot. K) for 30 min on ice. Digestion was stopped by adding 1 mM PMSF. Where indicated, mitochondria were converted to mitoplasts to selectively rupture the outer mitochondrial membrane. Mitochondria or mitoplasts were reisolated and resuspended in import buffer. All samples were finally precipitated with 10% trichloroacetic acid and analyzed by SDS-PAGE and fluorography. 10% STD, 10% of the amount of precursor added to each assay. (C) The first 47 amino acids of the Mcr1p precursor can direct import of attached DHFR into the outer mitochondrial membrane and into the intermembrane space. The precursor of Mcr1(1-47)-DHFR was synthesized in vitro, imported into isolated yeast mitochondria, and analyzed as described in the legend to Fig. 1A. The processed and unprocessed forms of Mcr1(1-47)-DHFR are indicated on the right. A second translation product, corresponding to mature-sized DHFR which is not imported into mitochondria, is indicated by an asterisk.

ergized mitochondria, about 15% of it was imported to a protease-protected location (Fig. 3B, lane 1). Import depended on an electrochemical potential across the inner membrane (Fig. 3B, lanes 1 and 2). About two-thirds of the imported molecules were protease protected in mitoplasts, indicating that they had completely crossed the inner membrane (Fig. 3B, lanes 3 and 4). As expected, the Mcr1(1-12)-DHFR fusion protein was not processed. Similar results were obtained for the import of native Mcr(1-12)-DHFR, although import was less efficient in this case (data not shown).

Import of Mcr1(1-47)-DHFR yielded the full-length protein as well as a processed form (Fig. 3C). About 20% of the fusion protein added to the import reaction became firmly inserted into the outer membrane and resistant to extraction at pH 11.5 (Fig. 3C, lanes 3 and 5). The remainder was apparently cleaved by inner membrane protease I and released into the intermembrane space (Fig. 3C, lanes 1 and 4) as a soluble protein which was released from the mitochondria upon rupturing of the outer membrane (Fig. 3C, lane 6). Generation of the smaller isoform required an electrochemical potential across the inner membrane (Fig. 3C, compare lanes 1 and 2). Cleavage of the fusion protein by inner membrane protease I was much less efficient than that of authentic Mcr1p (compare Fig. 1A and 3C).

We conclude that the first 12 amino acids of Mcr1p can act as a weak matrix-targeting signal (see also reference 21) and that the first 47 amino acids of Mcr1p can target an attached passenger protein to the outer membrane and the intermembrane space.

A double point mutation in the hydrophobic region of the targeting sequence of Mcr1p prevents insertion into the outer membrane and increases the efficiency of import into the intermembrane space. Which features of the targeting sequence are responsible for the dual intramitochondrial localization of Mcr1p? To answer this question we replaced alanines 23 and 24 in the middle of the hydrophobic putative membrane anchor domain with glutamines (Fig. 4A). The AAQQ mutant of Mcr1p had almost completely lost its ability to become firmly inserted into the outer membrane of isolated mitochondria (Fig. 4B, top panel) but was transported into the soluble intermembrane space even more efficiently than the wild-type precursor (Fig. 4B, bottom panel). Thus, weakening the hydrophobic character of the putative membrane anchor sequence prevents insertion into the outer membrane but increases import into the intermembrane space.

A 30-kDa fragment of the AAQQ mutant but not of the wild-type Mcr1p accumulates in the mitochondrial matrix. Some inner membrane proteins, such as subunit 9 of the F_0F_1-ATPase, are first translocated into the matrix and then inte-

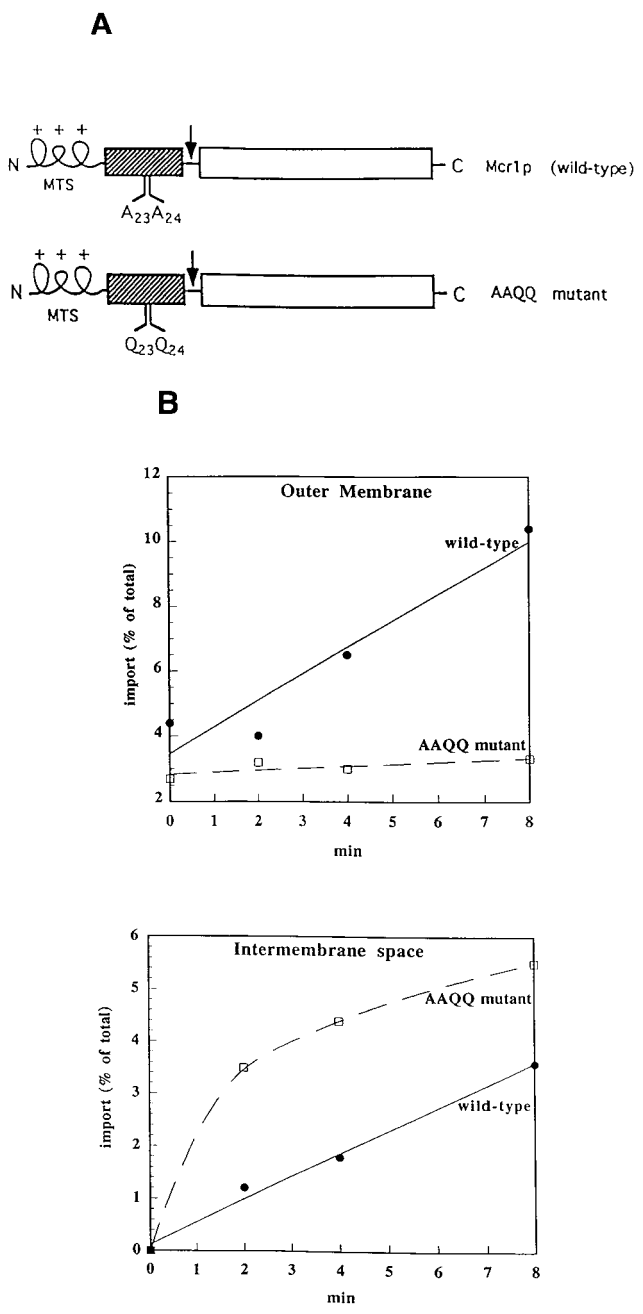

FIG. 4. A double point mutation in the hydrophobic region of the Mcr1p targeting sequence prevents insertion into the outer mitochondrial membrane and increases the efficiency of import into the intermembrane space. (A) Schematic representation of wild-type Mcr1p and the AAQQ mutant. Symbols are as explained in the legend to Fig. 3A. MTS, matrix-targeting sequence. The amino acid positions of the double point mutation are indicated. (B) Wild-type Mcr1p or the AAQQ mutant was synthesized in vitro and imported into fully energized mitochondria at 25°C for the indicated times. Insertion into the outer mitochondrial membrane was assayed by measuring the amount of carbonate-inextractable Mcr1(34) (upper panel). Import into the intermembrane space was assayed by quantifying the amount of protease-protected processed Mcr1(32) as described in the legend to Fig. 1 (lower panel). The amount of imported precursor [import (% of total)] is given as a percentage of the total amount of precursor added to each assay as a function of time (in minutes). Import experiments were carried out with the same batch of mitochondria and performed simultaneously.

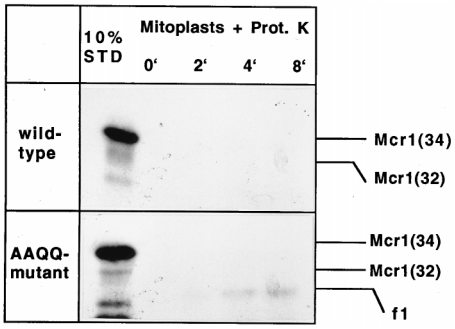

FIG. 5. A 30-kDa fragment of the AAQQ mutant, but not of the wild-type Mcr1 protein, accumulates in the mitochondrial matrix. Wild-type Mcr1p or the AAQQ mutant was synthesized in vitro and imported into fully energized mitochondria at 25°C for the indicated times (in minutes). Mitochondria were reisolated, converted to mitoplasts, and treated with proteinase K (Prot. K) for 30 min on ice. Digestion was stopped by adding 1 mM PMSF. The mitoplasts were reisolated, resuspended in import buffer, precipitated with trichloroacetic acid, and analyzed by SDS-PAGE and fluorography. 10% STD, 10% of the amount of precursor added to each assay; f1, 30-kDa protease-protected fragment.

grated into the inner membrane from the matrix side (26a). Others, such as the adenine nucleotide translocator (37) or subunit Va of cytochrome oxidase (22), insert directly into the inner membrane without passage through the matrix space. To address the question of how sorting of Mcr1p is achieved, we checked for the transient accumulation of Mcr1p in the mitochondrial matrix during import. We imported the wild-type Mcr1p or the AAQQ mutant into mitochondria and analyzed whether the imported molecules were protease protected in mitoplasts (Fig. 5). With the wild-type Mcr1p precursor, no such intermediate was detected (Fig. 5, top); by contrast, a small fraction of the AAQQ mutant of Mcr1p was missorted to the mitochondrial matrix, yielding a 30-kDa protease-protected fragment (Fig. 5, bottom).

This 30-kDa fragment appears to be unrelated to the 28-kDa band seen in Fig. 1A, which is readily digested by externally added proteinase K and hence represents nonimported protein nonspecifically bound to the mitochondrial surface (see also Fig. 1A). Generation of the 30-kDa fragment was linear with time (Fig. 5) and dependent on an electrochemical potential across the inner membrane and on ATP, indicating that it required an interaction of the precursor with the translocase in the mitochondrial inner membrane (data not shown). This fragment thus does not exhibit the properties of a true translocation intermediate but presumably represents a mistargeted "dead-end product" which had not completely crossed the mitochondrial inner membrane (see also reference 7).

Import of Mcr1p into the intermembrane space requires Tom7p. Previous work had suggested that sorting of Mcr1p to its final intramitochondrial destinations in vivo requires interaction of the Mcr1p precursor with the translocase of the mitochondrial outer membrane (10). Tom7p, a small subunit of the outer membrane translocase, has recently been identified to modulate the dynamics of the translocase and to play a role in the sorting and accumulation of preproteins at the outer membrane (14). We tested the effect of a *TOM7* deletion on the steady-state distribution of the two forms of Mcr1p. When total-cell lysates or isolated yeast mitochondria from the wild-type strain or an isogenic *TOM7* deletion strain were analyzed by SDS-PAGE and immunoblotting, both showed significantly reduced levels of Mcr1(32) and slightly increased levels of Mcr1(34) in the *TOM7* deletion strain (Fig. 6A and B). The levels of the mitochondrial marker proteins Tom22p, cyto-

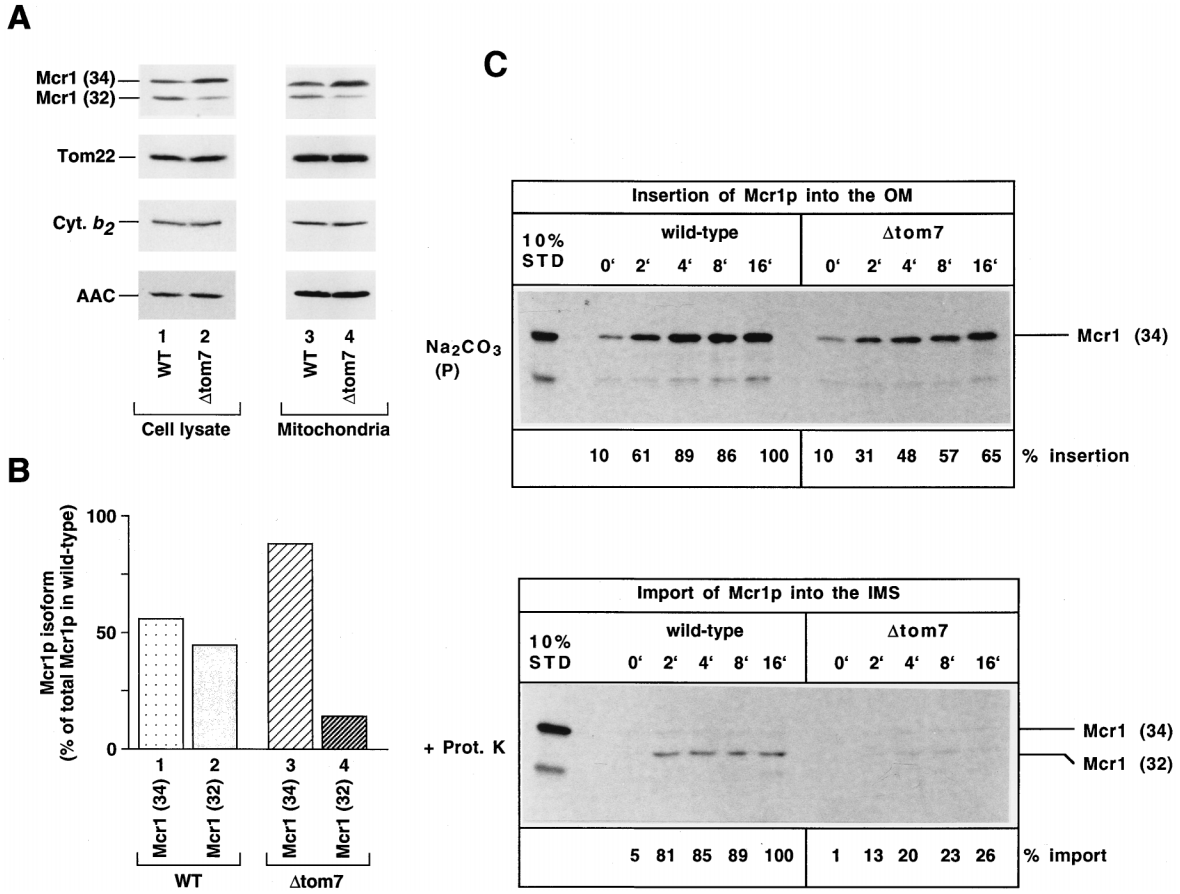

FIG. 6. Import of Mcr1p into the intermembrane space is strongly impaired in mitochondria lacking Tom7p. (A) Lack of Tom7p leads to an increase in Mcr1(34) and a decrease in Mcr1(32) in vivo. Cell lysates and mitochondria of wild-type (WT) *S. cerevisiae* and a Tom7p-less mutant (50 μg of protein) were prepared and immunoblotted with antisera monospecific for Tom22p, the ADP/ATP carrier (AAC), cytochrome b_2 (Cyt. b_2), and Mcr1p. Blots were developed with the ECL detection system (Amersham). The content of marker proteins (Tom22p, AAC, and cytochrome b_2) was indistinguishable between the wild type and the mutant. (B) The amounts of the two Mcr1p isoforms in wild-type and Δ*tom7* mitochondria were quantified by laser densitometry. The total amount of Mcr1p [Mrc1(32) plus Mcr1(34)] in wild-type mitochondria was taken as 100%. (C) Mcr1p was synthesized in vitro and imported into mitochondria from the wild-type or the Δ*tom7* deletion strain for the indicated times (in minutes). Under the conditions chosen, import was limited by the amount of mitochondria used in the assay. Insertion of Mcr1p into the outer mitochondrial membrane (OM) was assayed by measuring the amount of carbonate [Na_2CO_3 (P)]-inextractable Mcr1(34). Import of Mcr1p into the intermembrane space (IMS) was assayed by quantifying the amount of protease-protected (+ Prot. K) Mcr1(32) as described in the legend to Fig. 1. 10% STD, 10% of the amount of precursor added to each assay. Experiments with the wild-type and the Δ*tom7* mitochondria were performed simultaneously. The amount of imported Mcr1p was quantified by laser densitometry; relative amounts of imported Mcr1(34) and Mcr1(32) are given at the bottoms of the upper and lower panels, respectively. The amount of precursor imported into the outer membrane (upper panel) or into the intermembrane space (lower panel) of wild-type mitochondria after 16 min was taken as 100%.

chrome b_2, and the ADP/ATP carrier were identical in wild-type and mutant strains (Fig. 6A). The altered ratio of the two Mcr1p isoforms in the mutant mitochondria was also found upon importing the Mcr1p precursor into isolated mitochondria. Isolated mitochondria lacking Tom7p were unable to import the Mcr1p precursor into the intermembrane space (Fig. 6C, lower panel) but were only slightly less efficient in inserting Mcr1(34) into the outer mitochondrial membrane (Fig. 6C, upper panel). Tom7p is thus required for the efficient import of Mcr1p into the intermembrane space in vivo and in vitro, presumably by regulating exit from the outer membrane translocase.

Msp1p is another outer membrane protein with a putative function in intramitochondrial protein sorting (23). When we analyzed the steady-state distribution of Mcr1(34) and Mcr1 (32) in strains deleted for *msp1* or overexpressing Msp1p, we did not observe any significant differences compared to an isogenic wild-type strain (data not shown). Msp1p therefore does not appear to play an important role in the intramitochondrial sorting of Mcr1p.

Import of Mcr1p into the intermembrane space requires Tim11p. Tim11p is a recently discovered component of the inner membrane translocase which interacts efficiently with the intramitochondrial sorting signal of cytochrome b_2 (33a). To address the question whether Tim11p is also required for sorting of Mcr1p to the intermembrane space, we tested whether deletion of *TIM11* had any effect on the intramitochondrial levels of the two Mcr1p isoforms in vivo. Compared to wild-type mitochondria, mitochondria from a *TIM11* deletion mutant contained about 10-fold lower amounts of Mcr1(32), whereas the levels of Mcr1(34), of mhsp70 (a matrix protein), and of Tim44p (a peripheral inner membrane protein) were essentially unchanged (Fig. 7A and B). When wild-type and mutant mitochondria were analyzed for their ability to import Mcr1p in vitro, the Tim11p-less mitochondria were almost incapable of importing the Mcr1p precursor into the intermembrane space (Fig. 7C, lower panel) but were equally efficient in inserting it into the outer membrane (Fig. 7C, upper panel). Consistent with a role of Tim11p in mediating the sorting of preproteins within the inner membrane import site,

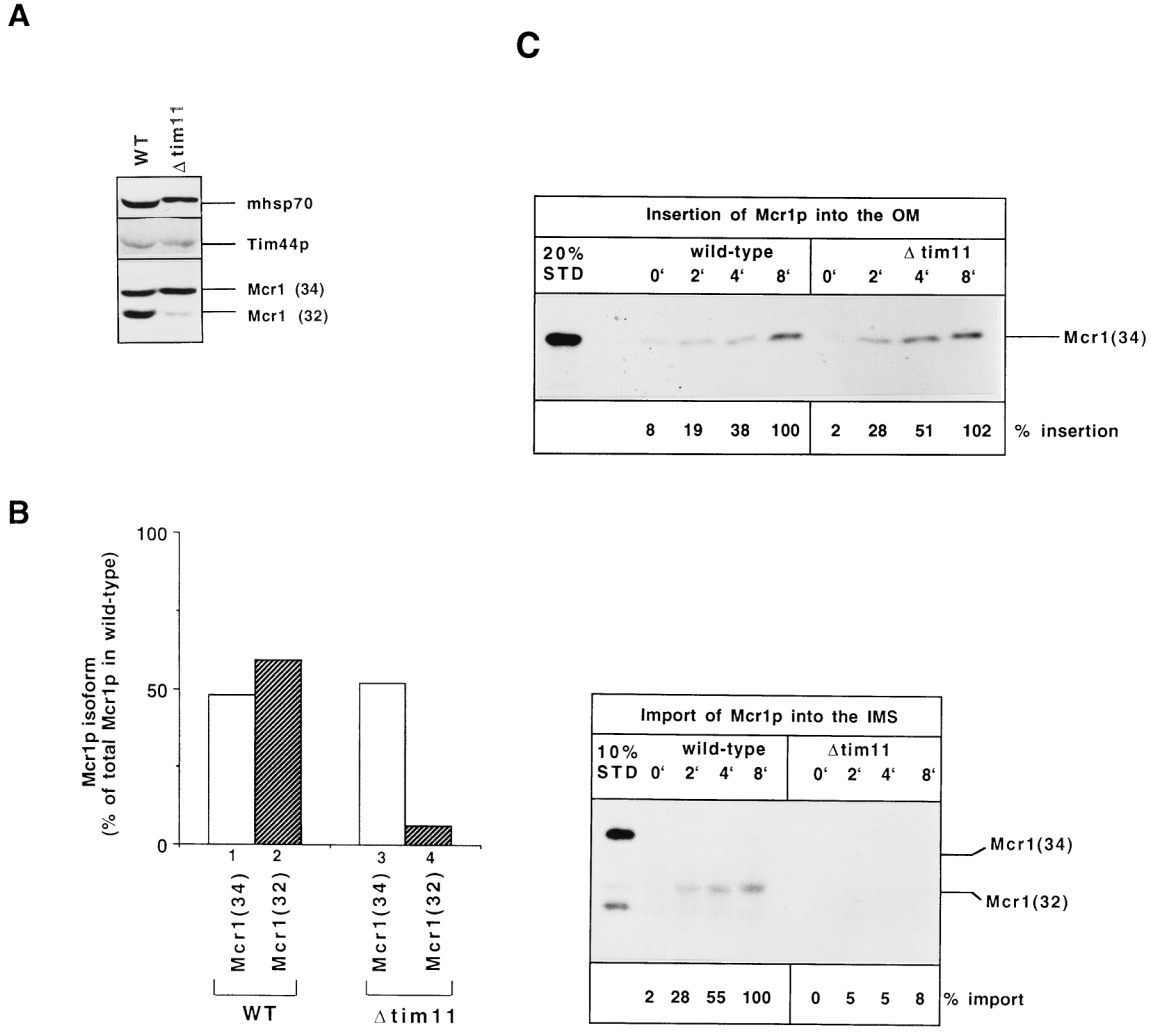

FIG. 7. Import of Mcr1p into the intermembrane space is strongly impaired in mitochondria lacking Tim11p. (A) Mitochondria lacking Tim11p contain significantly reduced amounts of Mcr1(32) compared to wild-type (WT) mitochondria. Mitochondria (100 μg of total protein) from the wild-type or the Δ*tim11* deletion strain were analyzed by SDS-PAGE and immunoblotting with antisera monospecific for mhsp70 (a matrix protein), Tim44p (an inner membrane protein), and Mcr1p. Blots were developed with ^{125}I-protein A and radioautography. The content of marker proteins (mhsp70 and Tim44p) was indistinguishable between the wild type and the mutant. (B) The amounts of the two Mcr1p isoforms in wild-type and Δ*tim11* mitochondria were quantified by laser densitometry. The total amount of Mcr1p [Mrc1(32) plus Mcr1(34)] in wild-type mitochondria was taken as 100%. (C) Mcr1p was synthesized in vitro and imported into mitochondria from the wild-type or the Δ*tim11* deletion strain for the indicated times (in minutes). Under the conditions chosen, import was limited by the amount of mitochondria used in the assay. Insertion of Mcr1p into the outer mitochondrial membrane (OM) was performed at 16°C and assayed by measuring the amount of carbonate [Na_2CO_3 (P)]-inextractable Mcr1(34) (upper panel). Import of Mcr1p into the intermembrane space (IMS) was assayed by quantifying the amount of protease-protected Mcr1(32) as described in the legend to Fig. 1 (lower panel). 10 or 20% STD, 10 or 20% of the amount of precursor added to each assay. Experiments with the wild-type and the Δ*tim11* mitochondria were performed simultaneously. The amount of imported Mcr1p was quantified by laser densitometry; relative amounts of imported Mcr1(34) and Mcr1(32) are given at the bottoms of the upper and lower panels, respectively. The amount of precursor imported into the outer membrane or into the intermembrane space of wild-type mitochondria after 16 min was taken as 100%.

we recently found that Tim11p-less mitochondria were also defective in importing the precursor of SU9-DHFR into the mitochondrial matrix (reference 33b and data not shown). We conclude that import of Mcr1p into the intermembrane space requires Tim11p and, hence, an interaction with the protein translocase in the inner membrane.

DISCUSSION

Mcr1p is synthesized with a putative N-terminal matrix-targeting signal, followed by 21 uncharged, mostly hydrophobic residues (10). This motif resembles the targeting signal found in the outer membrane import receptor Tom70p (12) but also bears similarity to the bipartite targeting signals of cytochrome b_2 and cytochrome c_1, two intermembrane space proteins (2a, 30, 35). In the present study we have shown that (i) the first 12 amino acids of this sequence can function as a weak matrix-targeting signal and (ii) the N-terminal 47 residues suffice to target an attached protein to the mitochondrial outer membrane and the soluble intermembrane space. The hydrophobic segment of the targeting sequence appears to be required for anchoring the protein to the outer membrane, as a double point mutation in this sequence abolishes insertion into the outer membrane without affecting import to the intermembrane space. Likewise, strengthening of the putative matrix-targeting signal by duplication of the first 12 amino acids of Mcr1p increases the efficiency of import into the intermembrane space (12a) and thus has an effect similar to that of the

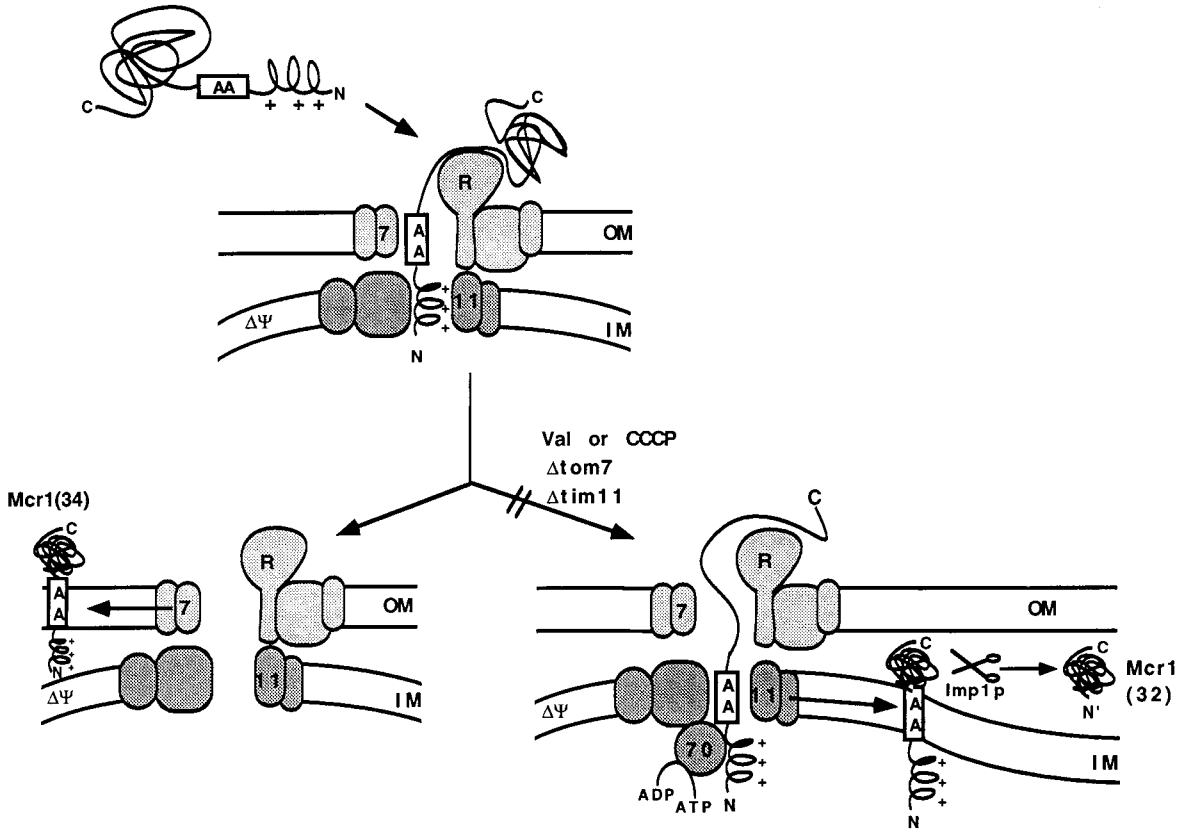

FIG. 8. Model for the import and sorting pathway of Mcr1p. See the text for an explanation. Abbreviations: OM, outer mitochondrial membrane; IM, inner mitochondrial membrane; R, protein import receptor; Imp1p, inner membrane protease I (depicted as scissors); Val, valinomycin, a K^+ ionophore; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone, a protonophore; ΔΨ, membrane potential. Numbers indicate the molecular weights of the depicted proteins (7, Tom7p; 11, Tim11p; 70, mhsp 70). Plus signs, positively charged amino acids.

double point mutation in the hydrophobic segment discussed above. These results strongly support our earlier hypothesis (10) that sorting of Mcr1p to the outer membrane and the intermembrane space is achieved by incomplete translocation arrest of the Mcr1p precursor in the outer membrane, and they are consistent with the following model (Fig. 8). Initially, the primary translation product interacts with the protein import receptor and then inserts into the outer membrane translocation machinery, as suggested by the inhibition of the import of both isoforms in trypsin-treated mitochondria (see Fig. 1B). We propose that already at this early step in translocation, the sorting pathways of both isoforms diverge (Fig. 8, top pathway). Transport of Mcr1p from the outer membrane translocase to the inner membrane requires Tom7p, a recently identified small subunit of this complex which appears to destabilize the interaction of the multisubunit protein import receptor (13, 19, 20) with the translocation pore (14); in the absence of Tom7p, most Mcr1p precursor molecules laterally escape from the outer membrane translocase and insert into the lipid bilayer of the outer membrane (Fig. 8, left pathway). Our results presented here and reported earlier (1, 14) suggest that the outer membrane translocase does not merely act as a passive diffusion pore that allows the random movement of a translocating chain (34) but instead may undergo conformational changes in order to actively promote anterograde transport to the inner membrane. Such a dynamic behavior has recently been shown for the translocase of the inner mitochondrial membrane (2).

Part of the Mcr1p precursor molecules do not become inserted into the mitochondrial outer membrane but instead cross that membrane, interact with the translocase of the mitochondrial inner membrane, and are finally sorted to the intermembrane space (Fig. 8, right pathway). As seen in vivo, the generation of the intermembrane space isoform of Mcr1p or the Mcr1(1-47)-DHFR fusion protein requires an electrochemical potential across the inner membrane; dissipating the membrane potential by use of the K^+ ionophore valinomycin or the protonophore carbonyl cyanide *m*-chlorophenylhydrazone completely inhibits the insertion of the precursor into the inner membrane translocase (Fig. 8). Stable insertion of the Mcr1p precursor into the inner membrane also requires an interaction with the inner membrane translocase and mhsp70 on the matrix side of the inner membrane (Fig. 8, right pathway). ATP hydrolysis in the matrix may provide the driving force for the unfolding and translocation of a tightly folded Mcr1p domain across the outer membrane. This proposal is supported by the observation that unfolding of the Mcr1p precursor by urea prior to import abolishes the requirement for matrix ATP and allows transport and processing in the absence of ATP. The energy requirements for importing Mcr1p to the intermembrane space therefore resemble those seen for import of cytochrome b_2 (9, 32).

Lateral movement of the precursor within the inner membrane requires the recognition of the hydrophobic sorting signal by Tim11p, a component identified by its efficient interaction with the sorting signal of cytochrome b_2 during passage

into the intermembrane space (33a). Unlike insertion into the outer membrane, the transient insertion of the protein into the inner membrane is not severely affected by the double point mutation in the hydrophobic stretch of the sorting signal, although in this case translocation arrest seems to be incomplete, as partially translocated molecules appear in the matrix (see Fig. 5). Finally, the inserted Mcr1p precursor is cleaved by inner membrane protease I and Mcr1(32) is released into the soluble intermembrane space (Fig. 8, right pathway). Some aspects of this model are still tentative, i.e., we do not know how gating of the import channels in the outer and inner membranes is achieved and how this contributes to the differential sorting of Mcr1p, but a more detailed biochemical analysis of this sorting process might help to answer these questions.

Moreover, it will be interesting to see whether other proteins that are differentially targeted to different intracellular locations or compartments within organelles make use of a similar mechanism.

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