FEBS Letters 582 (2008) 855–860

The outer membrane form of the mitochondrial protein Mcr1 follows a TOM-independent membrane insertion pathway

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Received 2 November 2007; revised 31 January 2008; accepted 6 February 2008

Available online 13 February 2008

Edited by Ulrike Kutay

Abstract **The yeast gene *MCR1* encodes two isoforms of the mitochondrial NADH-cytochrome *b*5 reductase. One form is embedded in the outer membrane whereas the other is located in the intermembrane space (IMS). In the present work we investigated the biogenesis of the outer membrane form. We demonstrate that while the IMS form crosses the outer membrane via the translocase of the outer mitochondrial membrane (TOM) complex, the other form is integrated into the outer membrane by a process that does not require any of the known import components at the outer membrane. Thus, the import pathways of the two forms diverge in a stage before the encounter with the TOM complex and their mechanism of biogenesis represents a unique example how to achieve dual localization within one organelle.**

Keywords: Mcr1; Mitochondria; Outer membrane; Protein import; Signal-anchored protein

1. Introduction

Most of the mitochondrial precursor proteins, especially those destined for the matrix are synthesized with an N-terminal extension, the presequence [1]. In contrast, all proteins of the mitochondrial outer membrane and some of the proteins destined to the inner membrane and the intermembrane space (IMS) are devoid of a typical presequence. These proteins contain non-cleavable targeting signals.

Mitochondria like all sub-cellular compartments and organelles contain a unique set of proteins that defines their biological function. However, it is well documented that a single eukaryotic gene can give rise to protein isoforms that are located in various subcellular locations [2]. The *Saccharomyces cerevisiae MCR1* gene represents yet a unique example of a gene with a single translation product which gets converted into two isoforms located in two different compartments within the same organelle. It encodes two mitochondrial isoforms of NADH-cytochrome *b*5 reductase: a 34 kDa isoform that is integrated into the outer membrane and a 32 kDa isoform located in the IMS [3]. The first 11 amino acid residues of Mcr1 can function in vitro as a rather ineffective presequence and are followed by 21 uncharged and mostly hydrophobic residues which can form a transmembrane segment (TMS) [4].

It was earlier suggested that the differential sorting of Mcr1 is achieved by a "leaky stop-transfer" mechanism. According to this model, all precursor molecules of Mcr1 are initially targeted to the translocase of the outer mitochondrial membrane (TOM complex) and the N-terminal domain of Mcr1 is inserted into the import pore of the translocase. Next, a fraction of the molecules is released into the membrane by lateral opening of the TOM complex giving rise to the 34 kDa form. The remaining molecules are further translocated across the outer membrane and interact at the inner membrane with the preprotein translocase of the inner membrane (TIM23 complex). Subsequently, the inner membrane protease 1 (Imp1) cleaves off the hydrophobic segment and the soluble 32 kDa form is released into the IMS [3,4]. Although this model is appealing, experimental evidence for lateral opening of the TOM complex and for an involvement of the latter in the membrane insertion of the 34 kDa form are missing.

The outer membrane form of Mcr1 has similar topology to a special class of mitochondrial outer membrane proteins, the so-called "signal-anchored" proteins. Proteins of this class like Tom20, Tom70 and OM45 contain a single transmembrane segment at their N-terminus [5,6]. We have previously found that the TOM complex is required for the biogenesis of newly synthesized Tom20 molecules [7]. However, since Tom20 is a subunit of the TOM complex, it might represent a special case that does not reflect the insertion pathways of other proteins from this class. In the present study we have investigated the mechanism by which the 34 kDa form of Mcr1 is inserted into the outer membrane. Unexpectedly, our results suggest that this isoform of Mcr1 follows a novel insertion pathway which is independent of the TOM complex.

2. Materials and methods

2.1. Yeast strains and growth conditions

Standard genetic techniques were used for growth and manipulation of yeast strains. The *S. cerevisiae* strain W303 was used. The *mcr1* null strain was constructed by replacing the *MCR1* open reading frame with the HIS3 cassette by homologous recombination. The *tom20* null strain YTJB64 and its corresponding parental strain YTJB4 were utilized

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Abbreviations: IASD, 4-acetamido-4′-[(iodoacetyl)amino]stilbene-2,2′-isulfonic acid; IMS, intermembrane space; TMS, transmembrane segment; TIM, translocase of the inner mitochondrial membrane; TOM, translocase of the outer mitochondrial membrane

0014-5793/$34.00
doi:10.1016/j.febslet.2008.02.009

(kind gift of Dr. G. Schatz [8]). Strains with *TOM40* temperature-sensitive alleles were obtained from Dr. Kassenbrock [9]. The Δ*tom70* strain was purchased from Research Genetics (Huntsville, AL).

2.2. Biochemical procedures

Mitochondria were isolated as described [10]. For isolation of mitochondria from temperature-sensitive and their parental strains, cells were grown at 25 °C. Radiolabelled precursor proteins were synthesized in rabbit reticulocyte lysate in the presence of [^{35}S]-methionine after in vitro transcription by SP6 polymerase from pGEM4 vectors containing the gene of interest. Blotting to nitrocellulose membranes and immunodecoration were performed according to standard procedures.

2.3. In vitro protein import and membrane insertion assay

Import experiments were performed in an import buffer containing 250 mM sucrose, 0.25 mg/ml BSA, 80 mM KC1, 5 mM $MgCl_2$, 10 mM MOPS–KOH, 2 mM NADH, 2 mM ATP, pH 7.2. Mitochondria isolated from the temperature-sensitive strain *tom40-3* or from the corresponding parental strain were incubated at 37 °C for 15 min before import reactions.

Labelling with IASD (4-acetamido-4′-[(iodoacetyl)amino]stil-bene-2,2′-disulfonic acid) was performed according to a published procedure with some modification [11]. Radiolabelled proteins were incubated for various time points at 25 °C with isolated mitochondria. Mitochondria were re-isolated by centrifugation and re-suspended in labelling buffer (0.6 M sorbitol, 20 mM HEPES–KOH, 50 mM Tris, 4 M urea, 1 mM DTT, pH 8.0) in the presence or absence of 5 mM IASD. In some samples Triton X-100 (1%) was added to the labelling buffer. All samples were incubated further for 20 min at 25 °C. The labelling reaction was stopped by adding 330 mM DTT and mitochondria were then re-isolated and resuspended in sample buffer.

2.4. Recombinant DNA techniques

The *MCR1* open reading frame was amplified by PCR from genomic yeast DNA. The resulting product was digested with EcoRI and HindIII and cloned into the EcoRI/HindIII sites of pGEM4 vector. The Mcr1 variants were constructed by site directed mutagenesis utilizing the Quick-Change Mutagenesis Kit (Stratagene). For expression in yeast cells the *MCR1* variants were introduced into the multicopy yeast plasmid, pYX132.

3. Results

3.1. Experimental design

To obtain a reliable assay for the membrane insertion of Mcr1 we employed a labelling procedure based on a specific modification of Cys residues by the membrane-impermeable sulfhydril-reactive reagent IASD. Modification with IASD has been used successfully to investigate the topology of other membrane proteins such as Bcl-2 and α-hemolysin [11,12]. Since IASD cannot enter the lipid core of the membrane, Cys residues can only be modified by this reagent when exposed to an aqueous environment. Therefore, the inability of IASD to label a Cys residue can be used as an indication of membrane insertion of the Cys-containing segment. The addition of one IASD (624 Da) molecule to a protein can be detected as a shift in the migration of the protein on SDS–PAGE. Consequently, an even larger shift is expected if two Cys residues within the same protein molecule are modified. Native Mcr1 contains a single Cys residue (Cys263) which is exposed to the cytosol (in the 34 kDa form) or to the IMS (in the 32 kDa form) (Fig. 1A). Thus, this residue can be labelled by IASD independently of the proper membrane insertion of the 34 kDa form. To study the membrane insertion of Mcr1 by an IASD-based labelling assay we created a Mcr1 variant with a single Cys residue within its putative TMS. To that end, a Thr-encoding codon (Thr19) in the putative TMS of Mcr1 was replaced by a Cys-encoding codon (Mcr1-T19C, Fig. 1A). This mutation resulted in a Mcr1 molecule with two Cys residues. Next, the Cys residue in position 263 was mutated to a Ser residue resulting in a Mcr1 molecule with a single Cys in its TMS (Mcr1-TMC, Fig. 1A).

We isolated crude mitochondria from Δ*mcr1* cells expressing plasmid-encoded native Mcr1 or one of the two Mcr1 variants described above. All Mcr1 variants were found in the crude mitochondria fraction, demonstrating that the mutations did not influence the capacity of Mcr1 to be imported into mitochondria (Fig. 1B). Next, we incubated the mitochondria isolated from the various strains with IASD. Of note, IASD can diffuse into the IMS via the pores formed in the outer membrane by the general transporter, porin. Therefore, proteins in the IMS can be modified upon incubation of intact mitochondria with IASD. Thus, as expected, both forms of native Mcr1 were found to be modified by IASD (Fig. 1C). A more complicated pattern was observed with the variant containing an additional Cys residue in the TMS (Mcr1-T19C). As with the native Mcr1 both the 32 and 34 kDa forms were modified with IASD (Fig. 1C). A further shift of the long form was observed upon lysis of the outer membrane with the detergent Triton X-100 since under these conditions both Cys residues are accessible to IASD. The fact that detergent-mediated solubilization was required to observe the second shift demonstrates that the Cys19 residue was originally protected from labelling due to its location within the membrane (Fig. 1C). A different picture emerged when the variant with the single Cys residue within the TMS domain (Mcr1-TMC) was analyzed. The 34 kDa form got modified only after solubilization of the membranes whereas the 32 kDa form of this variant does not contain any Cys residues and was therefore not modified (Fig. 1C).

To study the molecular mechanism of the membrane integration of the 34 kDa form we established an in vitro labelling assay by utilizing isolated mitochondria and radiolabelled Mcr1 variants synthesized in reticulocyte lysate. Since our assay is based on non-modification of membrane-embedded Cys residues as an indication for membrane insertion, we wanted to make sure that under our in vitro conditions the absence of external membranes results in an efficient labelling of the Cys residues. To that goal we incubated the radiolabelled precursors of the Mcr1 variants with IASD in the absence of mitochondria. Fig. 1D demonstrates that the vast majority of the molecules of the Mcr1 variants were indeed modified under these conditions. The very minute amount of molecules which was not completely labelled reflects most probably protection via membrane debris in the reticulocyte lysate since complete labelling was observed upon addition of detergent to the reaction mixture (Fig. 1D).

Next, radiolabelled precursors of Mcr1 were incubated with isolated mitochondria. We were not able to observe the 32 kDa form in this in vitro assay. As observed with Mcr1-T19C (and Mcr1(TMC), data not shown) the association kinetic is very fast (Fig. 1E). An initial incubation of 2 min followed by labelling with IASD resulted in a situation where the vast majority of the Cys19 residues were protected from modification and could only be modified upon addition of the detergent. Taken together, this labelling assay is Cys-specific and reflects correctly the insertion of the 34 kDa form into the mitochondrial outer membrane.

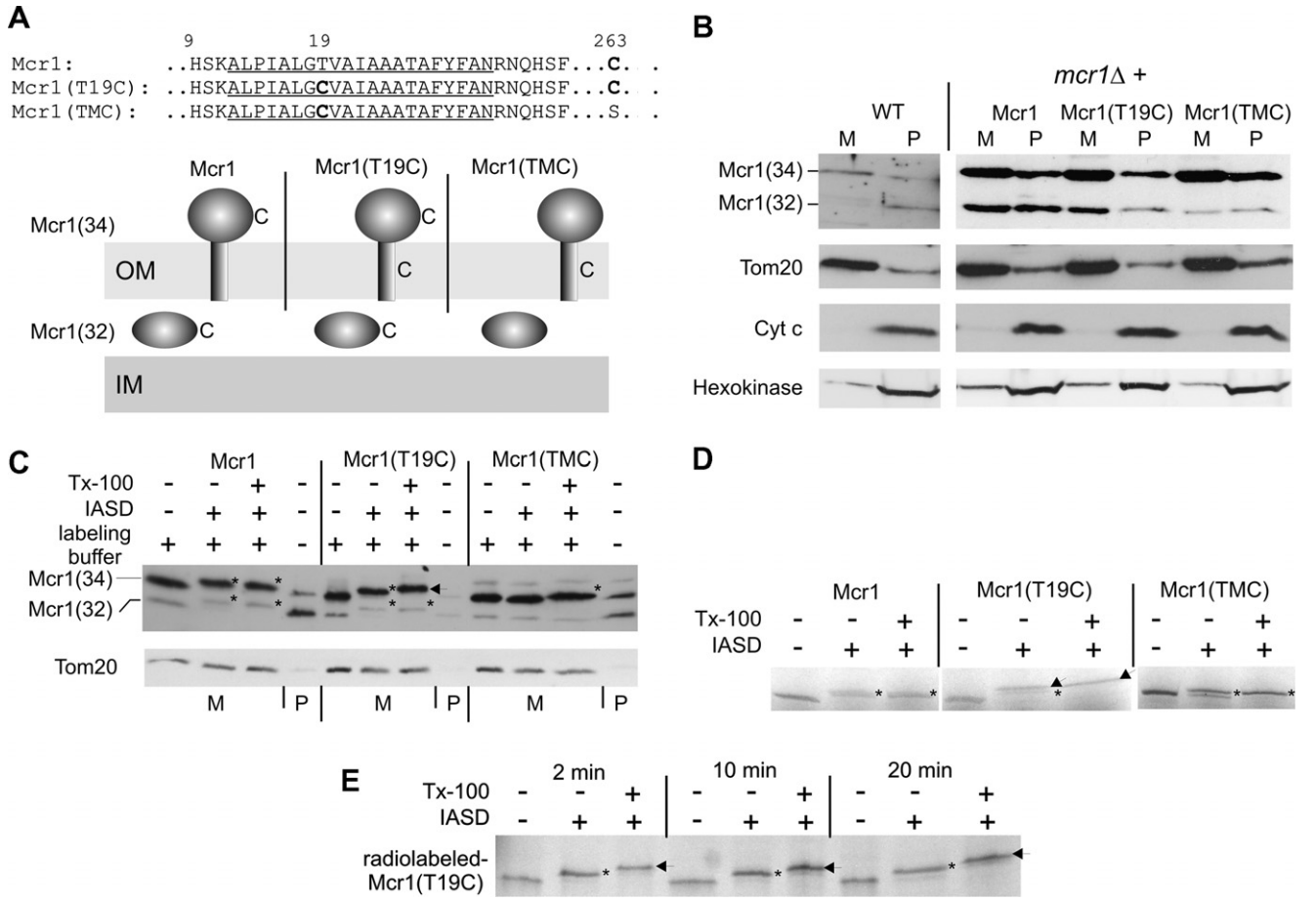

Fig. 1. Protection of Mcr1(TMC) from modification with IASD represents proper insertion into the outer membrane. (A) Upper panel: The sequences of the transmembrane domain of native Mcr1 and its variants are presented. Only relevant residues in the cytosolic domain are indicated. The putative transmembrane segment is underlined. Cys residues are in bold type. Lower panel: schematic representation of the two forms of native Mcr1 and its variants. (B) Mitochondrial and post-mitochondrial fractions (M and P, respectively) were obtained from wild-type (WT) and Δ*mcr1* cells transformed with an overexpressing vector encoding either authentic Mcr1 or the indicated Mcr1 variants. Samples were subjected to SDS–PAGE and immunoblotting with antibodies against Mcr1, the mitochondrial outer membrane protein Tom20, Cyt *c* which is located in the IMS, and hexokinase a marker protein for the cytosol. Of note, a fraction of outer membrane proteins and of the soluble IMS form of Mcr1 as well as the vast majority of the small soluble IMS protein Cyt c are found in the post-mitochondrial supernatant since the outer membrane of mitochondria gets ruptured during the procedure. (C) Crude mitochondria and a post-mitochondrial fractions were obtained as above (M and P, respectively). The crude mitochondria were incubated where indicated with IASD for 20 min in the presence or absence of Triton X-100 (Tx-100). Mitochondrial proteins were analyzed by SDS–PAGE and immunodecoration. Proteins modified by IASD at one Cys residue are indicated with an asterisk, whereas Mcr1(T19C) modified at both Cys residues is indicated with an arrowhead. (D) The indicated radiolabelled precursor proteins were incubated for 20 min in labelling buffer in the presence or absence of 1% Triton X-100 and IASD (5 mM). Samples were analyzed by SDS–PAGE and autoradiography. Proteins modified by IASD are indicated as in part C. (E) Radiolabelled precursor of Mcr1(T19C) was incubated with mitochondria for the indicated time periods. Mitochondria were centrifuged and resuspended in labelling buffer in the presence or absence of 1% Triton X-100 and IASD (5 mM). Samples were analyzed by SDS–PAGE and autoradiography. Proteins modified by IASD are indicated as in part C.

3.2. The insertion of Mcr1 into the outer membrane is independent of TOM import receptors

Using this in vitro assay we examined whether the import receptors, Tom20 and Tom70 are involved in the insertion of Mcr1 into the outer membrane. Removal of the exposed parts of surface receptors upon treatment of mitochondria with trypsin did not result in any reduction of the integration level of Mcr1. In contrast, as previously reported, the membrane integration of porin was strongly reduced upon this treatment ([13] and Fig. 2A). Next, mitochondria isolated from strains lacking either Tom20 or Tom70 were incubated with radiolabelled Mcr1(TMC). Both receptors appear not to be involved in the insertion of the 34 kDa form as their absence did not affect the insertion of this form into isolated mitochondria (Fig. 2B and unpublished results). As a control, the extent of membrane insertion of the porin precursor was significantly decreased when mitochondria lacking Tom20 were used (Fig. 2B). We further checked the steady-state levels of Mcr1 in the strains lacking the two import receptors. Whereas the 34 kDa form was observed in similar levels in all strains, lower amounts of the 32 kDa form were detected in the *tom20*Δ strain (Fig. 2C and D). Collectively, the import receptor Tom20 seems to mediate the import of the 32 kDa form into the IMS. However, none of the receptors appears to have any effect on the insertion of the outer membrane form. We further observed that the absence of other outer membrane proteins like the small Tom subunit Tom6 or of Mim1 which is involved in the biogenesis of the TOM complex did not influence the membrane insertion of Mcr1(34) (data not shown).

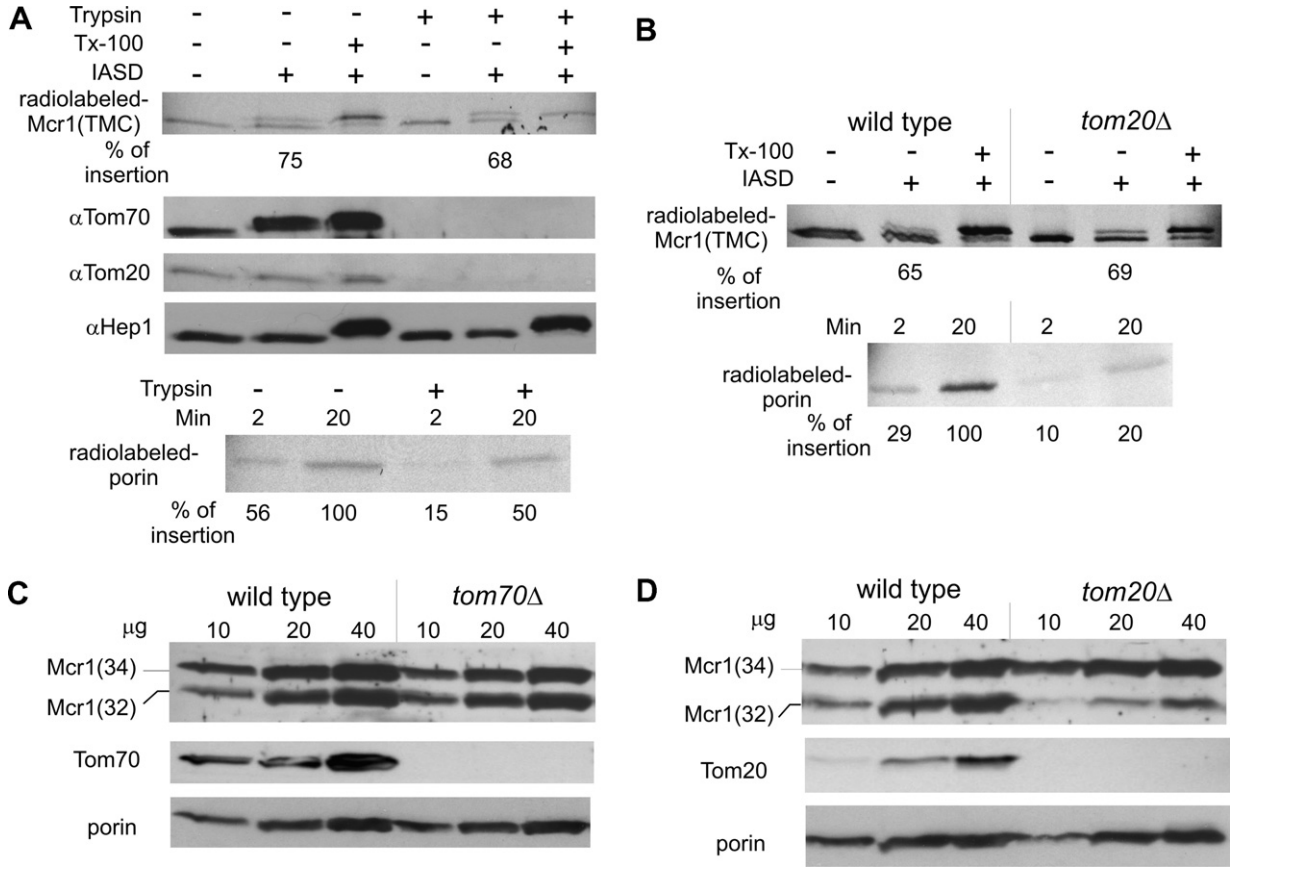

Fig. 2. The insertion of Mcr1 into the outer membrane does not require TOM import receptors. (A) Radiolabelled Mcr1(TMC) was incubated for 2 min with intact mitochondria or with mitochondria pretreated with trypsin. Modification with IASD was as described in the legend to Fig. 1C. Mitochondrial proteins were separated by SDS–PAGE, blotted to a membrane and then analyzed by autoradiography and immunodecoration with antibodies against the receptor proteins Tom20 and Tom70, and against the matrix protein Hep1 as a control. Insertion of Mcr1 is given as the fraction in % of bound material (−IASD) which is protected in the presence of IASD (lower band in +IASD). Lower panel: as a control, radiolabelled porin was incubated with intact or trypsin-pretreated mitochondria for the indicated time periods. Mitochondria where treated with proteinase K (PK) to degrade non-inserted protein molecules and mitochondrial proteins were analyzed by SDS–PAGE and autoradiography. The amount of porin precursor imported into intact mitochondria after 20 min was set to 100%. (B) Radiolabelled precursor of Mcr1(TMC) was incubated for 2 min with either wild-type mitochondria or with mitochondria isolated from cells lacking Tom20. Radiolabelled precursor of porin was incubated with the same mitochondria for the indicated time periods. Further treatment and quantification was as described in the legend to part A. (C, D) Mitochondria were isolated from wild-type or from cells deleted in either Tom70 (C) or Tom20 (D). The indicated amounts of mitochondria were analyzed by SDS–PAGE and immunodecoration with antibodies against mitochondrial proteins.

3.3. The TOM import pore is not involved in the membrane insertion of Mcr1

Beta-barrel precursors are translocated through the import pore of the TOM complex prior to their insertion into the outer membrane [14,15]. Thus, we investigated whether the import pore of the TOM complex plays any role in the insertion of Mcr1. To address this question we added to the import reaction an excess amount of recombinant presequence-containing precursor protein which is known to use the import channel (pSu9(1-69)-DHFR) [16]. Blocking the import channel did not influence the insertion of radiolabelled Mcr1, whereas the import of porin was strongly reduced (Fig. 3A). We further tested the insertion capacity of mitochondria harbouring the *tom40-3* temperature-sensitive allele of Tom40. Mitochondria containing this Tom40 variant show an impaired import of presequence-containing precursor proteins [9,17]. Mitochondria isolated from this mutated strain could insert the 34 kDa form with efficiency similar to that of mitochondria isolated from the corresponding parental strain (Fig. 3B). Of note, similar steady-state levels of the 34 kDa form were observed in both types of mitochondria. In contrast, the 32 kDa form was expressed in reduced amounts in the mitochondria containing the Tom40-3 variant (Fig. 3C). Taken together, mutations in the TOM complex affect the biogenesis of the 32 kDa form similarly to their effect on the import of presequence-containing precursor proteins. In contrast, these mutations do not have any influence on the membrane insertion of the 34 kDa form.

3.4. Positively-charged residues in the N-terminal region are not crucial for the insertion of the outer membrane form

A canonical mitochondrial presequence which is recognized by the TOM complex has a net positive charge [18]. Similarly, the presequence-like segment of Mcr1 contains three basic residues (Fig. 4A). Are these residues important for the dual-localization of the protein? To that end we mutated both Arg residues at positions 4 and 7 to Glu residues resulting in a segment with a neutral net charge (Mcr1(TMC)R4E,R7E,

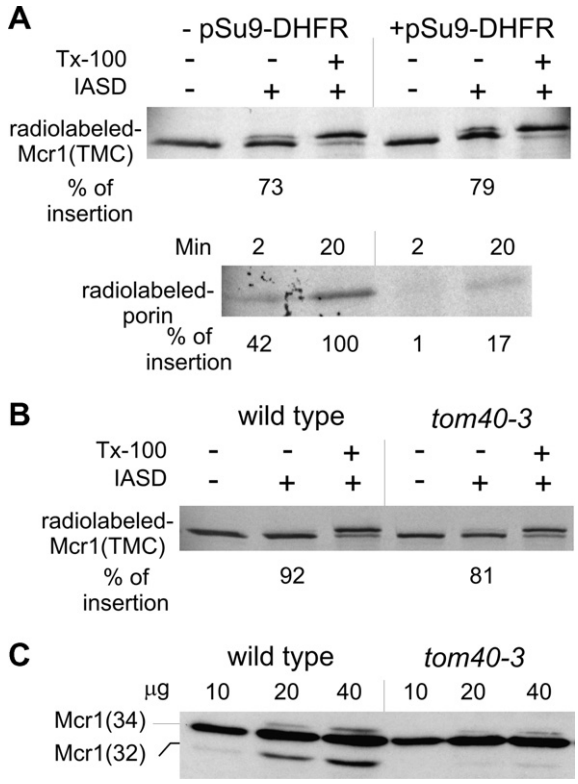

Fig. 3. The TOM import pore is not involved in the membrane insertion of Mcr1. (A) Radiolabelled precursor of Mcr1(TMC) was incubated for 2 min with isolated mitochondria in the presence or absence of excess recombinant pSu9-DHFR. Radiolabelled precursor of porin was incubated with the same mitochondria for the indicated time periods. Further modification with IASD and analysis of import was as described in the legend to Fig. 2A. (B) Radiolabelled precursor of Mcr1(TMC) was incubated for 2 min with wild-type or *tom40*-3 mitochondria. Further treatment was as described in the legend to Fig. 2A. (C) Various amounts of mitochondria as in (B) were analyzed by SDS–PAGE and immunodecoration with antibodies against Mcr1.

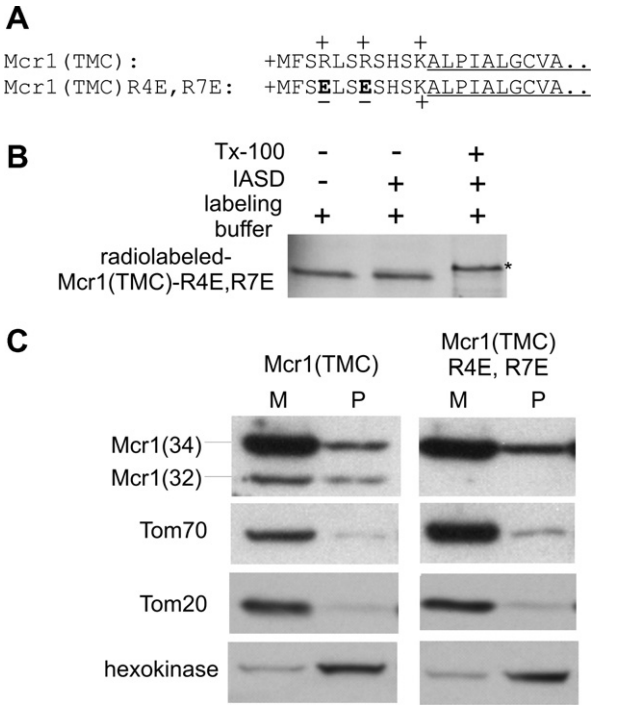

Fig. 4. Positively-charged segment at the N-terminal domain of Mcr1 is not required for the membrane insertion of the 34 kDa form. (A) The sequences of the N-terminal domain of Mcr1(TMC) and its charge variant are presented. The putative transmembrane segment is underlined and charges of the residues are indicated. Mutated residues in the variant protein are in bold type. (B) Radiolabelled precursor of Mcr1(TMC)-R4E,R7E was incubated with mitochondria for 2 min. Further treatment was as described in the legend to Fig. 1E. (C) Mitochondrial and post-mitochondrial fractions (M and P, respectively) were obtained from Δ*mcr1* cells transformed with a vector encoding either Mcr1(TMC) or Mcr1(TMC)-R4E,R7E. Samples were subjected to SDS–PAGE and immunoblotting with antibodies against Mcr1, Tom20, Tom70, and the cytosolic protein, hexokinase.

Fig. 4A). We tested then the in vitro insertion of this variant and observed that its membrane integration is as efficient as that of the native protein (Fig. 4B). Next, yeast cells were transformed with a plasmid expressing Mcr1(TMC)R4E,R7E and we monitored the steady-state levels of both Mcr1 forms. The outer membrane form of this charge variant was present at similar amount to the outer membrane form of the Mcr1 variant with a positively-charged presequence-like region (Fig. 4C). In sharp contrast, hardly any molecules of the short form of Mcr1(TMC)R4E,R7E were observed (Fig. 4C). Taken together, these experiments provide further evidence to the notion that the outer membrane form of Mcr1 is integrated into the outer membrane by a unique pathway, which does not share elements with the general import pathway.

4. Discussion

Mcr1 provides a rare example of a protein with a dual-localization within the same organelle. Our results support the previously proposed pathway for the biogenesis of the 32 kDa IMS form of Mcr1 [3,4]. According to this model the presequence-like segment of Mcr1 is recognized by the TOM complex before being translocated via the import pore into the IMS. Next, Mcr1 molecules interact with the TIM23 complex and Imp1 processes then the precursor and releases the 32 kDa soluble form into the IMS. Similar import pathways of precursors with bipartite signals are known for other mitochondrial proteins like cytochrome b_2, CCPO and Mgm1 [19].

Less clear is the insertion pathway of the outer membrane form of Mcr1. According to the "leaky stop-transfer" model the divergence in the import pathways of the 32 and the 34 kDa forms occurs due to incomplete arrest at the TOM complex. Whereas one form continues from the TOM complex to the IMS to be processed later on, the other form was suggested to be arrested at the TOM complex before a lateral opening of the latter releases this form into the lipid core of the membrane [3,4]. A lateral opening of the TOM complex however, was not observed so far and is under debate due to thermodynamic considerations [21]. Furthermore, our current results suggest that the TOM complex is not involved in the biogenesis of the 34 kDa form of Mcr1. Such a TOM-independent insertion pathway into the mitochondrial outer membrane was proposed recently for proteins containing a single membrane anchor segment at their C-terminal domain (tail-anchored proteins) ([22] and Kemper, unpublished results). Further support for our proposal are the observations made

by us and others, that mutations in Tom40 result in a reduced amount of the 32 kDa form, but in normal levels of the outer membrane form [23].

The TOM-independent pathway does not seem to be common for all "signal-anchored" proteins. We have found that the import pore of the TOM complex is not required for the insertion of Tom70 and OM45. However, the TOM complex is involved in the membrane insertion of newly synthesized Tom20 molecules [7]. As Tom20 is a subunit of the TOM complex it is not surprising that the correct topology of Tom20 depends on preexisting TOM complexes. Mim1 is another example of an outer membrane protein which promotes the insertion of Tom20 into the outer membrane [24] but is not involved in the membrane integration of Mcr1. Thus, Tom20 might represent a specific case, whereas Mcr1 follows probably a more general insertion pathway.

We observed that a Mcr1 variant with a neutral net charge in its N-terminal flanking region could still be inserted into the outer membrane. Similarly, variants of other signal-anchored proteins, Tom20 and OM45, with neutral charge in the N-terminal flanking region of the TMS were able to integrate into the outer membrane [20]. Hence, whereas positively-charged residues are crucial for the function of a peptide as a canonical presequence, they are not essential for the functionality of a signal-anchor domain.

In summary, we propose that the integration of Mcr1 into the mitochondrial outer membrane occurs via a novel pathway, which does not involve the known import components in the mitochondrial outer membrane. Obviously, cytosolic factors can facilitate the specific delivery of Mcr1 precursor molecules to mitochondria.

Acknowledgements: We thank K. Rehn and E. Kracker for technical assistance, C. Koehler and K. Hell for providing antibodies, and K.S. Dimmer for helpful discussions. This work was supported by Sonderforschungsbereich 446-A30 and Grant RA1028/2-1 of the Deutsche Forschungsgemeinschaft.

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