The bacterial magnesium transporter CorA can functionally substitute for its putative homologue Mrs2p in the yeast inner mitochondrial membrane

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The yeast nuclear gene MRS2 encodes a protein of 54 kDa, the presence of which has been shown to be essential for the splicing of group II intron RNA in mitochondria and, independently, for the maintenance of a functional respiratory system. Here we show that the MRS2 gene product (Mrs2p) is an integral protein of the inner mitochondrial membrane. It appears to be inserted into this membrane by virtue of two neighboring membrane spanning domains in its carboxyl-terminal half. A large amino-terminal and a shorter carboxyl-terminal part are likely to be exposed to the matrix space. Structural features and a short sequence motif indicate that Mrs2p may be related to the bacterial CorA Mg$^{2+}$ transporter. In fact, overexpression of the CorA gene in yeast partially suppresses the pet$^-$ phenotype of an mrs2 disrupted yeast strain. Disruption of the MRS2 gene leads to a significant decrease in total magnesium content of mitochondria which is compensated for by the overexpression of the CorA gene. Mutants lacking or overproducing Mrs2p exhibit phenotypes consistent with the involvement of Mrs2p in mitochondrial Mg$^{2+}$ homeostasis.

Magnesium is the most abundant divalent cation within living cells, and it serves as an essential cofactor in innumerable enzymatic reactions. It has equally important roles in maintaining tertiary structures of proteins and RNAs, and it plays an important role in the catalysis of ribozymes and in RNA splicing (1–4). Failure to maintain appropriate levels of metal ions in humans may be a feature of hereditary and acquired diseases (3–6). In eukaryotic cells magnesium concentrations are in the lower millimolar range and they are relatively constant when extracellular concentrations are altered dramatically. Polyphosphate, RNA, and ATP bind most of the intracellular magnesium, leaving only a small fraction as free, ionized Mg$^{2+}$. This pool of the free ion is presumed to enter biochemical processes and to be transported in the form of magnesium ion (4, 7, 8). Although many observations indicate that transport systems for magnesium do exist, molecular studies on transporters are still scarce.

CorA, an integral protein of the bacterial plasma membrane, has been shown to be the constitutive magnesium transporter in prokaryotes, mediating both uptake and efflux (9, 10). In the yeast Saccharomyces cerevisiae three putative homologues of the CorA protein have been recently noticed. Functional studies of yeast overexpressing or lacking one of these genes, named ALR1, are consistent with a role in the transport of magnesium and cobalt through the yeast plasma membrane (11).

Here we present a detailed study on the location and possible function of the Mrs2 protein which is another putative homologue of the CorA family in S. cerevisiae. The nuclear MRS2 gene had been isolated in a search for suppressors of mitochondrial defects and was shown to play a role in RNA splicing and other functions in mitochondria (12). The data reveal that Mrs2p is inserted into the inner mitochondrial membrane, putatively via two membrane spanning domains, and exposes large parts to the matrix space. Like the putative cytoplasmic transporter Alr1p in yeast (11), the mitochondrial Mrs2 protein has weak similarity to the bacterial CorA protein family of magnesium ion transporters (9, 10). Partial suppression of the petite phenotype of a mrs2 disruption mutant by the overproduction of the CorA protein in yeast suggests that CorA and Mrs2 proteins may be functionally related. This notion is supported by our finding of decreased or increased magnesium contents of mitochondria in strains lacking or overproducing Mrs2p, respectively.

MATERIALS AND METHODS

Strains, Plasmids, and Media—Escherichia coli strains were cultivated at 37 °C in LB medium and supplemented with 100 µg/ml ampicillin when appropriate. E. coli strains DH5α (Stratagene) and HMS174DE3 (F–, recA, rK12, mK12+, Rfr+) (Novagen) and the following bacterial and yeast plasmids were used for subcloning: pET22b(+) (Novagen), YEp551 (17), YCplac22, Yiplac211, and YCplac33 (18), and pVT-103-U (19). Yeast strains, all with group I and group II introns in their mtDNA, growth media, and conditions used were as described previously (12–15). In order to disrupt the ALR1 gene, a disruption cassette was PCR1-amplified by use of the pJJ244-URA3 cassette (16) and oligonucleotide primers of sequences flanking the ALR1 gene (11): ALR1-S1 5′-AAAGATCATGATATTGCTGAAAAGCGTAAAAGCATTAGTGTTTCCCG-3′ and ALR1-S2 5′-TCCTGGACTTTAAATCTTTCTACACATCATACCATCAGTCGCTGGCACAGACAGGTTTCCCG-3′. The PCR product obtained was transformed into the yeast strain GA74–1A, and Ura+ colonies were selected. Correct replacement of the ALR1 open reading frame (alleles alr1-1) by the disruption construct was verified by analytical PCR.

For chromosomal integration of an HA-tagged MRS2 allele (see below), the plasmid Yip-MRS2-HA was linearized by AporI digestion and transformed into strain DBY747/mrs2-1A followed by selection for Ura+ transformants. Correct integration was verified by analytical PCR and functional complementation of the pet phenotype of the recipient strain.

Anti-Mrs2 Serum—A 1.4-kilobase pair NdeI BamHI PCR fragment of the MRS2 gene, comprising the complete open reading frame (12),
was cloned in frame into the NdeI/BamHI sites of pET22b (+) (Novagen) to generate plasmid pET/MRS2, expressing the MRS2 protein under the control of the T7 promoter and with a carboxyl-terminal 6×His tag. Bacteria transformed with pET/MRS2, expressing the MRS2 protein, was found to express a novel protein of 54 kDa, which is in accordance with the expected molecular mass of the His-tagged MRS2 protein. This recombinant protein was purified from *E. coli* lysates by virtue of its ability to bind specifically to Ni2+–nitrilotriacetic acid-agarose, according to the manufacturer’s protocol (Qiagen), and was used for the immunization of rabbits. The serum was found to react with the novel protein of 54 kDa of recombinant bacteria.

**Plasmid Constructs**—To generate an *MRS*-HA construct, the *MRS2* gene was amplified by the polymerase chain reaction from the plasmid YEpl5/MRS2 (12), using the mutagenic oligonucleotide primers M21 and M2II, 5′AAAGGATCCGACGACGCAGC5′ and 3′MRS2-HA (CAGCAGCTTGTATACC3′), respectively, relative to the MRS2 coding sequence (12), and introducing BamHI and NolI sites (underlined). The product was cloned into the BamHI site of the plasmids YEpl5/MRS2, YIpcl211, and YCpal33, yielding the plasmids YEpl5MRS2mut, YIp1211M2mut, and YCpal33M2mut. A cassette coding for a triple hemagglutinin (HA) epitope tag was cloned in frame into the *MRS2* gene at the NolI site of the plasmids YEpl5MRS2mut, YIp53M2mut, and YCpal33M2mut. The resulting plasmids, named YEpl5MRS2HA, YIp53MRS2HA, and YCpal33M2HA, were able to complement the *pet* phenotype of the strain DBY747Msrs2a-1.

To obtain an MRS2-DHFR fusion construct, the BamHI-EcoRI fragment of the DHFR open reading frame was isolated from plasmid pDS5 (21), and the MRS2-containing plasmid YEpl5MRS2 (12) was cleaved at a single Asol site near the 3′ end of the *MRS2* gene. The ends were made blunt with Klenow fragment and dTTPs and ligated in frame to yield plasmid YEpl5MRS2-DHFR.

To express the bacterial CorA protein (9) in yeast, the respective gene was amplified by the polymerase chain reaction from the genomic DNA of the *Salmonella typhimurium* LT2 strain, using the oligonucleotide primers 5′AAACTCCACGCGGGATCTGATGCCGAGTTTCAACG3′ and 5′AAACGGATCCCAAGTTTCGATCTGAATCC3′; the product was cloned into the *SacI* site of the plasmids YEpl5/MRS2, YEpl5/MRS2/EcoRI, YEpl5/MRS2/XbaI, and YEpl5/MRS2/BamHI, introducing *SacI*, *EcoRI*, *XbaI*, and *BamHI* sites, respectively. The result was a 54-kDa novel protein of 54 kDa, which is in accordance with the expected molecular mass of the His-tagged MRS2 protein. This recombinant protein was purified from *E. coli* lysates by virtue of its ability to bind specifically to Ni2+–nitrilotriacetic acid-agarose, according to the manufacturer’s protocol (Qiagen), and was used for the immunization of rabbits. The serum was found to react with the novel protein of 54 kDa of recombinant bacteria.

**Miscellaneous**—The following procedures were performed essentially according to published methods (15): manipulation of nucleic acids, DNA sequencing, preparation of yeast protein extracts, separation of proteins on sodium dodecyl sulfate-polyacrylamide gels, immunoblotting, immunodetection, and computer analysis.

**RESULTS**

**Localization of MRS2p in Mitochondria**—To determine the subcellular location of the MRS2 gene product, we raised an antisera against purified, His-tagged MRS2p expressed heterologously in *E. coli* (see “Materials and Methods”). This antisera specifically reacted with a protein of 54 kDa in a mitochondrial fraction of yeast cells overproducing MRS2p from a multicopy plasmid, whereas no signal was detected in untransformed strains expressing wild-type levels of MRS2p only (Fig. 1A). HA-tagged MRS2p was detected by a monoclonal hemagglutinin antibody as a faint band of the expected size in a mitochondrial fraction when expressed from a single copy chromosomal gene and as a very strong band when expressed from a multicopy plasmid (Fig. 1B).

To verify that the 54-kDa protein was indeed MRS2p, we transformed yeast cells with a multicopy plasmid encoding an MRS2-DHFR fusion protein with a predicted molecular mass of 65 kDa (see “Materials and Methods”). The MRS2p antisera was found to react with a protein of the expected size in a mitochondrial fraction, verifying the specificity of this serum (Fig. 1A).

When mitochondria were fragmented into pellet and supernatant after treatment with alkaline Na2CO3, the MRS2 protein was found in the membrane fraction as was the integral membrane protein Aac2p (Fig. 1C). As expected, the soluble protein cytochrome *b*2 (intermembrane space) and the membrane-associated subunit α of the *F*1-ATPase were found in the supernatant. This indicated that MRS2p is (partially) integrated into a mitochondrial membrane.

After further fractionation of mitochondria, MRS2p was enriched in the inner mitochondrial membrane as was the carrier protein Aac2p (Fig. 1D). Porin and cytochrome *b*2 were enriched in the outer membrane and in the intermembrane space, respectively. The contact site fraction is expected to be composed of both outer and inner mitochondrial membranes (22), and accordingly, MRS2p, Aac2p, and porin were detected in this fraction. The presence of porin in the intermembrane space and of cytochrome *b*2 in the outer membrane fraction is indicative of incomplete separation of these fractions.

**Orientation of MRS2p in the Mitochondrial Inner Membrane**—After partial separation of the outer mitochondrial membrane, the resulting “mitoplasts” were treated with proteinase *K* (Fig. 2A). Hsp60p, a protein of the mitochondrial matrix, remained protected in degradations by proteinase *K*, indicating that the mitoplasts stayed intact. Aac2p, an integral inner membrane protein, shifted to a product of slightly higher electrophoretic mobility, indicating that the outer membrane was disrupted and that the short amino-terminal part of Aac2p, which is exposed on the outer side of the mitochondrial inner membrane (25), was accessible to proteinase *K* degrada-
Mrs2p Is a Putative Mg\(^{2+}\) Transporter in Yeast Mitochondria

**Fig. 1. Subcellular localization of Mrs2p.** A, specificity of an anti-Mrs2p serum. Mitochondrial proteins were solubilized, separated on a 10% SDS-polyacrylamide gel, and immunodecorated with anti-Mrs2p serum. Mitochondria were isolated from the following GA74–1A strains: wild-type (Wt), mrs2-1 disruptant (\(\text{mrs2}^{-}\)), wild-type transformed with multi-copy plasmid carrying either the MRS2 gene (YEpMRS2) or the MRS2-DHFR fusion (YEpMRS2-DHFR). B, comparison of Mrs2p expression levels. HA-tagged Mrs2p was expressed from a single chromosomally integrated copy (YIpMRS2-HA) or from a multi-copy episomal plasmid (YEpMRS2-HA) in strain DBY747.}

**D. M. Bui, J. Gregan, E. Jarosch, A. Ragnini, and R. J. Schweyen, unpublished results.**
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![Fig. 3. Topology of Mrs2p and its similarity to CorA and Alr1 proteins. A, hydropathy profile of Mrs2p (top) and its proposed orientation in the mitochondrial inner membrane (bottom). Calculations were performed as described previously (27) using the indices given and a span setting of 11. Putative membrane spanning domains are indicated by two bars labeled 1 and 2 (top) or by shaded boxes (bottom). Net charges of the inter-domain sequence and the carboxyl terminus, respectively are indicated. IMS, intermembrane space; IM, inner mitochondrial membrane. B, sequences of membrane spanning domains and flanking regions of the Mrs2, CorA, and Alr1 proteins. Characteristic features of the predicted membrane spanning domains and flanking sequences are highlighted. + and − indicate positive and negative charges, respectively. Amino acids of the conserved motif (Y/F/GMN) are underlined.](image)

YEpl51 (not shown). The CorA protein lacking the aminoterminal Mrs2 peptide is preferentially detected in the extramitochondrial fraction, and it does not exert any significant suppression of the mrs2-1 Δ mutant (Fig. 4).

A hallmark of the mrs2-1 Δ mutant phenotype is the nearly complete block in splicing of group II introns (12). As shown by RT-PCR assays in Fig. 4B, the exon-exon junction product and the exon-intron junction product are amplified exclusively from wild-type and from mrs2-1 Δ mutant RNA, respectively. Upon expression of the Mrs2-CorA fusion protein in the mrs2-1 Δ mutant, however, both products are amplified. Expression of this fusion protein thus restores RNA splicing of group II intron a15c to a considerable extent. The ratio of the two products stayed the same when the number of PCR cycles was varied (not shown). Taken together, these results indicate that the CorA protein can partially substitute for Mrs2p, provided that because influx of this ion is limited (11). In strain GA74-1A, Mg\textsuperscript{2+} concentrations of 20 mM are sufficient to restore growth of alr1-1 Δ mutant cells on fermentable and on non-fermentable substrate (Fig. 5). When disrupted in the MRS2 gene (mrs2-1 Δ) strain GA74-1A (12) can grow on complete media with a non-fermentable carbon source (Fig. 5). The alr1-1 Δ, mrs2-1 Δ double disruptant, however, exhibits a synthetic phenotype with strongly reduced growth on non-fermentable substrates with 20 mM Mg\textsuperscript{2+}. Increased concentrations (120 mM Mg\textsuperscript{2+}) lead to a partial suppression of this growth defect on non-fermentable substrate. On fermentable substrates growth is partially restored by the lower concentration and completely restored by the higher concentration of Mg\textsuperscript{2+} (Fig. 5). This effect is not observed with elevated concentrations of other divalent metal ions (Ca\textsuperscript{2+}, Co\textsuperscript{2+}, Fe\textsuperscript{2+}, Mn\textsuperscript{2+}, Ni\textsuperscript{2+}, Zn\textsuperscript{2+} and Cu\textsuperscript{2+} and data not shown). In an alr1-1 Δ mutant background of strain GA74-1A the mrs2-1 Δ mutation thus confers a conditional pet−
phenotype, dependent on the Mg$^{2+}$ concentration in the growth medium.

**Altered Mitochondrial Mg$^{2+}$ Concentrations**—Extracts of mitochondrial fractions were prepared from various strains (Table I), and Mg$^{2+}$ concentrations in these fractions were determined as described under “Materials and Methods.” Mitochondria of a strain disrupted in the MRS2 gene contain significantly lower and those of an MRS2-overproducing strain contain significantly higher Mg$^{2+}$ concentrations than mitochondria from wild-type cells. Overexpression of the MRS2/CorA fusion protein in an mrs2-disrupted strain leads to the restoration of nearly wild-type levels of Mg$^{2+}$. Mitochondria of a respiratory-deficient strain, mit$^{-}$ M1301, were found to contain similar Mg$^{2+}$ concentrations as mitochondria of wild-type cells. This indicates that the reduced content in mitochondria of mrs2 cells is not simply due to their respiratory deficiency. Absence or overexpression of Mrs2p had no significant effect on concentrations of other ions in mitochondria (Table I) nor on Mg$^{2+}$ and other cation concentrations in total cell extracts (data not shown).

**DISCUSSION**

The MRS2 gene product is unique among the factors supporting RNA-catalyzed splicing in that its presence is essential for the splicing of all four group II introns in yeast mitochondria, and without intron-encoded maturation (12). Here we characterize Mrs2p as an integral protein of the inner mitochondrial membrane. Structural and functional similarity to the bacterial magnesium ion transporter CorA as well as measurements of ion concentrations in mitochondrial extracts indicate that Mrs2p is involved in mitochondrial metal ion homeostasis. These findings open new routes toward a better understanding of the role of metal ions in mitochondria.

Mrs2p co-purifies with proteins of the inner mitochondrial membrane, and it is resistant to extraction by alkaline carbonate, just like other integral proteins of this membrane. Treatment of mitoplasts with proteinase K does not change the apparent electrophoretic mobility of Mrs2p nor does it degrade a hemagglutinin epitope tag added to the carboxyl terminus of Mrs2p. The data presented here indicate that Mrs2p is inserted into the inner mitochondrial membrane, putatively via two transmembrane domains, and that both hydrophilic ends are oriented toward the matrix side.

Similarity between proteins of the Mrs2 family and the bacterial magnesium ion transporter CorA, although essentially restricted to the presence of two predicted membrane spanning domains in their carboxyl termini, a short sequence motif in one of them and charge distributions (cf. Fig. 3), hints at a possible function of Mrs2p in mitochondrial metal ion homeostasis. The yeast homologues Alr1p and Alr2p, putative components of the magnesium ion transport system in the plasma membrane, and a related protein, Mrn2 (11), are the only yeast proteins in the data banks that also exhibit these similarities to CorA, indicating that they may be typical for a highly diverged family of metal ion transporters.

Strong support for a function of Mrs2p in mitochondrial metal ion homeostasis comes from our observation that the pet phenotype of a yeast strain lacking Mrs2p is suppressed by the CorA protein when it is efficiently expressed in yeast and targeted to mitochondrial membranes. Apparently, the bacterial magnesium ion transporter can either substitute for Mrs2p or otherwise compensate for the absence of the yeast protein. The fact that its expression partially overcomes the group II intron splicing defect is in favor of a true substitution of Mrs2p by the CorA protein.

Homologous functions of Mrs2 and CorA proteins are further suggested by the effects which their overexpression or mutation have in bacteria and in yeast. First, their absence increases the dependence of cells on high Mg$^{2+}$ concentrations in growth media, although only when mrs2 or CorA mutants are combined with other mutations affecting uptake of this ion (see Refs. 9 and 10 and this work). The dependence of mrs2 mutants on high Mg$^{2+}$ concentrations is particularly pronounced on non-fermentable substrate, suggesting a function of Mrs2p in mitochondrial Mg$^{2+}$ homeostasis. Second, their expression from multi-copy plasmids selectively decreases tolerance to the toxic metal ions Co$^{2+}$ and Ni$^{2+}$, whereas disruption of the MRS2 gene or mutation of the CorA gene leads to increased tolerance of cellular growth to these ions (9, 10). Co$^{2+}$ and Ni$^{2+}$ are likely to be taken up by the magnesium ion transporters CorA and Alr1 (9–11). A role of Mrs2p in mitochondrial magnesium homeostasis is suggested by our finding of changes in mitochondrial total magnesium concentrations which increases with overexpression of Mrs2p and decreases upon disruption of the MRS2 gene. The CorA protein, expressed in yeast and targeted to the mitochondria, compensates for the absence of Mrs2p in that it restores wild-type levels of mitochondrial magnesium.

A transport or regulatory function of the MRS2 gene product

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**FIG. 5. Synthetic pet$^{-}$ phenotype of the alr1-1,Δ, mrs2-1,Δ double mutant.** Serial dilutions of wild-type and mutant cultures of yeast strain GA74-1A were grown for 5 days at 28 °C on complex media with non-fermentable substrate (YPG) or with fermentable substrate (YPD) to which 40 and 120 mM MgCl$_2$ were added as indicated. WT, wild-type; Δmrs2, mutant with the mrs2-1,Δ allele; Δalr1, mutant with the alr1-1,Δ allele; Δmrs2Δalr1, double mutant.

**Table I**

| Ion concentrations of mitochondrial extracts from wild-type and mutant DBY747 yeast cells |
|---------------------------------------------------------------|
| | MRS2 | YEp | M1301 | Δmrs2 | Δmrs2-pVTU-MRS2/CorA-HA |
| | nmol/mg protein | nmol/mg protein | nmol/mg protein |
| DBY-YEp-MRS2 | 281.56 ± 19.83 | 18.05 ± 7.67 | 854.95 ± 104.42 |
| DBY-YEp | 194.14 ± 4.77 | 26.45 ± 2.81 | 807.23 ± 140.54 |
| DBY-M1301 | 185.07 ± 4.75 | 22.84 ± 5.86 | 814.60 ± 130.33 |
| DBY-Δmrs2 | 95.56 ± 12.31 | 28.10 ± 3.25 | 850.46 ± 130.10 |
| DBY-Δmrs2-pVTU-MRS2/CorA-HA | 184.24 ± 6.45 | 18.34 ± 5.19 | 910.55 ± 147.27 |
in mitochondrial Mg\textsuperscript{2+} homeostasis is consistent with our previous findings on its effects on group II intron RNA splicing (12); Mg\textsuperscript{2+} plays a prominent role in both the formation of tertiary structures and in the catalytic reactions of ribozymes (1, 2). The observed block of group II intron RNA splicing in mrs2-1 \textit{D} mutant cells of strain DBY747 (12) may be explained by the observed reduction in mitochondrial Mg\textsuperscript{2+} concentrations, whereas suppression of the splicing defect of an intron mutation (\textit{mit} \textsuperscript{+} M1301) (12, 13) could be due to the observed increase in mitochondrial magnesium concentration which results from overproduction of Mrs2p. This notion is supported by our observations that high Mg\textsuperscript{2+} concentrations in the medium enhance this suppression \textit{in vivo} (not shown) and partially restore self-splicing of M1301 mutant RNA \textit{in vitro}.\textsuperscript{2} Moreover, the absence of Mrs2p is compensated for by Mrs3p, Mrs4p, or Mrs12p, three members of the mitochondrial carrier family, and we have speculated previously that they may be involved in the transport of cations (14, 28).

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