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The G-M-N motif determines ion selectivity in the yeast magnesium channel Mrs2p

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The highly conserved G-M-N motif of the CorA-Mrs2-Alr1 family of Mg^{2+} channels has been shown to be essential for Mg^{2+} transport. We performed random mutagenesis of the G-M-N sequence of *Saccharomyces cerevisiae* Mrs2p in an unbiased genetic screen. A large number of mutants still capable of Mg^{2+} influx, albeit below the wild-type level, were generated. Growth complementation assays, performed in media supplemented with Ca^{2+} or Co^{2+} or Mn^{2+} or Zn^{2+} at varying concentrations, lead to identification of mutants with reduced growth in the presence of Mn^{2+} and Zn^{2+}. We hereby conclude that (1) at least two, but predominantly all three amino acids of the G-M-N motif must be replaced by certain combinations of other amino acids to remain functional, (2) replacement of any single amino acid within the G-M-N motif always impairs the function of Mrs2p, and (3) we show that the G-M-N motif determines ion selectivity, likely in concurrence with the negatively charged loop at the entrance of the channel thereby forming the Mrs2p selectivity filter.

Introduction

Magnesium is required for numerous cellular processes involved in cell architecture, its growth, energy metabolism and replication.[1–5] Maintenance of Mg^{2+} homeostasis is therefore critical for cell viability. Since cellular membranes are impermeable to Mg^{2+}, involvement of various Mg^{2+}-transport mechanisms is required to mediate Mg^{2+} influx or efflux *via* plasmalemma and biomembranes forming subcellular compartments.

Members of the large, heterogeneous CorA/Mrs2/Alr1 protein superfamily are found in prokaryotes and in both lower and higher eukaryotes (including *regnum* of plants). In bacteria (*Salmonella enterica* ser. *typhimurium*), yeast (*Saccharomyces cerevisiae*) and mitochondria of higher eukaryotes they were characterised as being the high-affinity Mg^{2+} uptake systems. They enable growth of bacteria and yeast in media provided even with very low Mg^{2+} concentrations.[6–11] Mutants lacking transporters/channels of the CorA/Mrs2/Alr1 superfamily cannot survive without being provided with highly concentrated Mg^{2+} in the surrounding environment.[12–16]

The *MRS2* gene encodes a 54 kDa integral protein of the inner mitochondrial membrane (Mrs2p). Yeast cells lacking *MRS2* are respiratory deficient and therefore exhibit a growth defect on non-fermentable substrates (''*petite* phenotype'').[12–16] Besides Mrs2p, *S. cerevisiae* expresses a homologous protein known as Mfm1p/Lpe10p, essential for magnesium homeostasis and group II intron splicing in yeast.[14] Deletion of *MFM1* also results in a ''*petite* phenotype''[17] and in a considerable reduction of the mitochondrial membrane potential ($\Delta\Psi$).[18–20]

The three-dimensional crystal structure of the bacterial Mg^{2+} channel CorA (a distant relative of Mrs2p) has already been solved.[18–20] Conservation of the primary sequences in the CorA/Mrs2/Alr1 protein superfamily is in the range of 15–20%.[6,8] Despite the low primary sequence homology there are several conserved features. These are in particular the two α-helices, named ''willow helices'', in the large N-terminal part and two trans-membrane helices (TM1, TM2) near the C-terminus connected by a short conformationally flexible loop.[17] The sequence G-M-N, a motif at the end of TM1, and the presence of bulky hydrophobic amino acids in the predicted gate region at the intracellular (intramitochondrial) end of the pore are the only universally conserved features, indicating an essential role of these amino acid residues in the function of CorA/Mrs2/Alr1 proteins.[18–20]

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By using mag-fura-2 it was shown that Mrs2p behaves as a channel mediating rapid and tightly regulated Mg^{2+} uptake into mitochondria.[9] Isolated mitochondria respond to a rise in the external magnesium concentration ($[Mg^{2+}]_e$) with a rapid increase of the mitochondrial free Mg^{2+} concentration ($[Mg^{2+}]_m$) equivalent to 25% per s relative to the basal $[Mg^{2+}]_m$ value.[9] Utilizing patch-clamp, Mrs2p was confirmed to be a Mg^{2+} channel with a conductance of ~150 pS.[21] Similarly high Mg^{2+} conductances have been reported by Moomaw and Maguire for CorA (*S. typhimurium*).[22] The assumption of a common mechanism of Mg^{2+} transport for both, Mrs2p and CorA, is further supported by the fact that Mrs2p can functionally substitute for CorA[17] and *vice versa* (this study). Furthermore, Mg^{2+} transport is in both cases inhibited by cobalt(III)-hexammine, a coordinated analogue of the hexahydrated Mg^{2+} ion.[21,23,24]

Mrs2p has been shown to be permeable also for Ni^{2+}, however, with a 3.5-fold lower conductance (~45 pS) compared to Mg^{2+}.[21] It is not permeable for Ca^{2+}, Mn^{2+} or Co^{2+}.[21] Additionally, suppression of Mg^{2+} currents in the presence of Co^{2+} was observed, suggesting Co^{2+} to interact with the pore.[21] This is different to *S. typhimurium* CorA and yeast Alr1p for which transport of Ni^{2+} as well as of Co^{2+} has been reported.[6,23]

The G-M-N motif has been shown to be critical for the function of CorA and even conservative single amino acid mutations completely abolish Mg^{2+} transport.[25,26] This was also confirmed for Mrs2p.[9] This suggests that this sequence is essential for the function, possibly through suitable positioning of the periplasmic loop implicated in the initial interaction with the hydrated Mg^{2+} [18] or/and in assisting in stripping off the hydration shell.[22,27,28]

In previous attempts to obtain a crystal structure of *Tm*CorA, high concentrations of Mg^{2+} or Ca^{2+} were used, trapping the protein in its supposable closed state. Very recently, Pfoh *et al.* have identified a *Tm*CorA mutant that can be crystallized in the presence or absence of Mg^{2+}.[27] Furthermore, also the crystal structure of *M. jannaschii* CorA, displaying the extracellular loop, has been published.[28] Analyses of these two structures have led to new models how conformational changes mediate opening and closing of the channel and how ion selectivity is achieved.[27,28] In the structure obtained by Pfoh *et al.* in the presence of Mg^{2+}, one ion was found to be coordinated by G and N of the G-M-N motif. The electron density at a distance of 4 Å from the carbonyl group of glycine and the asparagine side chain of the G-M-N motif was interpreted as a bound hydrated Mg^{2+} ion.[27] Similarly, a hydrated Mg^{2+} ion coordinated by the side chain of the asparagine residue of the G-M-N motif was observed in the structure of *M. jannaschii* CorA.[28] The fact that in both structures a Mg^{2+} ion is coordinated by the G-M-N motif supports the concept that this sequence is crucial for the initial interaction of the channel with its substrate and for ion selectivity.[27,28]

In order to further investigate the importance and role of the G-M-N motif we performed random PCR mutagenesis on the G-M-N triplet to obtain mutants harbouring all possible amino acid combinations and identified those still capable of transporting Mg^{2+}. The active mutants were further characterized using *in vivo* and *in vitro* studies showing that the G-M-N motif can be in part functionally replaced by certain combinations of amino acids. Our results corroborate the notion that this motif plays an important role in ion selectivity.

Results

Screening of triple G-M-N mutants

According to the studies of Szegedy and Maguire[25] and Kolisek and colleagues,[9] single amino acid substitutions in the G-M-N motif of CorA or Mrs2p are sufficient to abolish Mg^{2+} transport. Since single conservative mutations in the G-M-N motif are poorly tolerated, we performed a triple site random mutagenesis screen in order to address the question whether any other amino acid combination can substitute for this unique and universally conserved motif.

Since large-scale isolation of mitochondria from yeast is extremely time consuming and thus not suitable for high throughput analyses, we developed a bacteria-based system to screen for functional G-M-N mutants. Based on the ability of Mrs2p to functionally substitute for its bacterial homologue CorA,[17] we assumed that Mrs2p expressed in *Salmonella* strain MM281 depleted of all major Mg^{2+} transport systems could complement the Mg^{2+} transport deficiency. *Salmonella* strain MM1927, lacking the magnesium uptake systems MgtA and MgtB but over-expressing the Mg^{2+} channel CorA, considered to be the major Mg^{2+} uptake system in *Salmonella*, was used as the positive control. Growth of MM281 cells is only supported at high magnesium concentrations ranging from 10 (as shown in Fig. 1) to 100 mmol l^{-1}. MM1927 and MM281 cells expressing Mrs2p grow equally well without addition of external $MgCl_2$. This clearly proved the ability of Mrs2p to complement the *corAΔmgtAΔmgtBΔ* induced Mg^{2+} dependent growth defect of strain MM281 and enabled us to investigate *de novo* generated G-M-N mutants of Mrs2p at the functional level in bacteria.

After transformation of the mutant library into strain MM281, we replica plated the transformants on non-Mg^{2+}-supplemented LB plates containing 0.05 mmol l^{-1} IPTG to induce protein expression. These growth-restricting conditions allowed solely mutants still capable of Mg^{2+}-transport to proliferate.

We obtained a considerable amount of G-M-N mutants able to grow without additional Mg^{2+} supplementation. Out of those 62 mutants were sequenced. In 7 of them the G-M-N motif remained intact, outlining the functional dominance of this sequence. The remaining 55 mutants contained mutations of the G-M-N sequence. In 6 cases we obtained identical mutations in duplicates leaving us with 49 various mutations (Table 1). These 49 mutants were further analyzed using a growth complementation assay on plates supplemented with different IPTG concentrations to investigate how their ability to transport Mg^{2+} differs from wild-type Mrs2p. The assays were scored with symbols from "++++", for cells able to grow at each of the respective dilution steps to "−" meaning no growth (Table 1).

Bacterial cells transformed with wild-type *MRS2* were able to grow well on plates supplemented with only 0.03 mmol l^{-1}

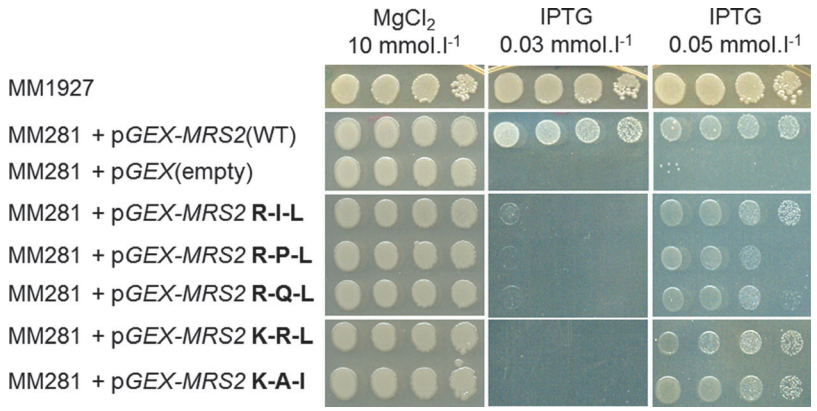

Fig. 1 Representative examples of the growth complementation of the MM281 mutant strain by Mrs2p and its selected G-M-N mutants. Over-night cultures of MM1927 and MM281 were transformed with plasmids indicated, serially diluted and spotted on LB medium plates supplemented with 10 mmol l^{-1} magnesium chloride or 0.03 and 0.05 mmol l^{-1} IPTG and incubated at 37 °C for 24 hours.

IPTG (Fig. 1). In contrast, the analyzed mutants exhibited only poor growth at this IPTG concentration or did not grow at all (examples are shown in Fig. 1). With increased IPTG concentrations (0.035–0.05 mmol l^{-1}) viability and growth of most of the mutants improved significantly (Table 1 and Fig. 1). These results showed that several (predominantly) G-M-N triple and some double mutants are still capable of mediating Mg^{2+}-influx albeit at lower efficiency than wild-type Mrs2p. The lower transport activity of the functional mutants is reflected by the higher expression levels needed for growth complementation in most of the mutants.

Sequences of the functional mutants

The prominent feature of the amino acid sequences of functional mutants is their divergence from the canonical G-M-N motif. A glycine at the first position is observed only twice, a methionine appears twice at the second position, and in only one case there is an asparagine at the third position. The only mutant resembling the wild type protein is characterized by the presence of a G-T-N tripeptide instead of G-M-N.

Interestingly, about 80% of the functional mutants have a positively charged residue in the G-M-N motif. In 59% of the cases this occurs at the first position, in 18% of the cases at the second position, and only in 4% of the cases at the third position. However, the co-presence of two positively charged amino acids is rather uncommon – only in about 10% of the cases. There is only one evident correlation between two positions; in 59% of the cases in which the first position is occupied by a positively charged residue, a hydrophobic amino acid (Val, Ile or Leu) occupies the third position. In contrast, a few negatively charged residues are observed in functional mutants, *i.e.* only six times in the first position and twice in the other two positions. No other clear trends were observed.

In a previous mutational analysis of the G-M-N motif of *S. typhimurium* CorA, none, even the most conservative single amino acid substitutions (A-M-N, G-A-N, G-C-N, G-I-N, G-M-A, G-M-L, G-M-Q), were tolerated.[25] A mutational study of Mrs2p performed by Kolisek and coworkers[9] also confirmed the importance of the G-M-N motif; mutation to A-M-N reduced Mg^{2+} uptake to the level of the *mrs2Δ* mutant. These findings are in agreement with our results, as neither these mutants nor any other single amino acid mutation – with the exception of the rather poorly growing G-T-N mutant – were found amongst the functional mutants, which are notably diverse from the native motif G-M-N.

Therefore, it might be concluded that while single substitutions within the canonical G-M-N motif are evolutionarily not tolerated, multiple adjacent mutations, though unlikely to occur in nature, result in functional molecules.

The effect of mutations of the G-M-N sequence on Mrs2p cation selectivity

We performed a growth complementation assay on plates containing 0.05 mmol l^{-1} IPTG and different concentrations of Ca^{2+} or Co^{2+} or Mn^{2+} or Zn^{2+}. These ions are known to be transduced by the yeast plasma membrane Mg^{2+} channel Alr1p, a homologue of Mrs2p.[29] We selected 10 mutants for this assay (Tables 2 and 3, Fig. 2), in which we examined if the presence of the aforementioned divalent cations in the growth medium influenced cell growth compared to wild-type Mrs2p.

Seven of the chosen mutants had a positively charged amino acid (K, R, H) at the first position and a small, hydrophobic residue at the third position (like approx. 35% of the mutants of Table 1). The last three mutants did not fit in the pattern "positively charged – X – hydrophobic", and were chosen as representatives of well (R-A-W), medium (R-R-T) and poorly (R-V-H) complementing mutant variants.

In the case of Ca^{2+} and Co^{2+} we did not observe any difference (results not shown) in the growth complementation assay compared to non-supplemented plates, suggesting that no influx or blockage of the channel by these ions occurred. In a study employing the patch clamp technique on giant lipid vesicles fused with the inner-mitochondrial membrane, Mrs2p was permeable for Mg^{2+} and Ni^{2+} but not for Ca^{2+}, Mn^{2+} or Co^{2+}. *Per contra* suppression of Mg^{2+} currents in the presence of Co^{2+} was seen.[21] In our case no effect of Co^{2+} was observed, both on

Table 1 *S. typhimurium* strain MM281 was transformed with p*GEX-Mrs2*(WT) or p*GEX* carrying various *Mrs2* G-M-N mutants (as indicated), serially diluted and replica plated on plates with increasing IPTG concentrations (0.03–0.05 mmol l^{-1}). The number of ''+'' symbols corresponds to the number of dilution steps exhibiting growth

		IPTG (mmol l^{-1})				
No.	Sequence	0.03	0.035	0.04	0.045	0.05
1	G-M-N	++++	++++	++++	++++	++++
2	R-F-V[a]	+	++++	++++	++++	++++
3	R-I-L	+	++++	++++	++++	++++
4	R-M-V	+	++++	++++	++++	++++
5	R-Q-I	+	++++	++++	++++	++++
6	R-Q-L[a]	+	++++	++++	++++	++++
7	R-V-L[a]	+	++++	++++	++++	++++
8	L-R-C	++	+++	+++	++++	++++
9	R-A-W	+	++++	+++	++++	++++
10	R-V-M	+	++++	+++	++++	++++
11	I-R-I	++	+++	++	++++	++++
12	K-A-I	–	++++	+++	++++	++++
13	K-R-L	–	++++	+++	++++	++++
14	R-F-I	+	++++	+++	++++	+++
15	R-V-I	+	++++	+++	++++	+++
16	R-N-L	–	+++	+++	++++	++++
17	R-P-L	+	+++	+++	++++	+++
18	C-F-L	++	++	++	+++	++++
19	R-G-F	+	+++	+++	+++	+++
20	R-S-V	+	+++	+++	++++	++
21	R-C-V	–	++	++	++++	++++
22	F-R-L[a]	+	++	++	+++	+++
23	D-F-G	+	++	+	+++	+++
24	K-A-M	–	++	++	+++	+++
25	R-F-Y	+	++	++	+++	++
26	R-R-T	–	++	++	+++	+++
27	V-R-A	+	++	++	++	+++
28	V-R-C	+	++	++	++	+++
29	D-F-P[a]	+	++	+	++	+++
30	E-F-P	+	++	++	++	++
31	K-H-V	–	++	++	+++	++
32	E-Q-V	+	++	+	++	++
33	G-D-M	+	++	+	++	++
34	K-Y-I	–	++	++	++	++
35	R-T-Y	–	++	++	++	++
36	E-F-A	+	+	+	++	++
37	G-T-N	+	+	+	++	++
38	K-M-L	–	++	+	++	++
39	P-D-L[a]	–	+	++	++	++
40	R-F-Q	–	++	+	++	++
41	R-V-H	–	+	+	+++	++
42	R-Y-S	–	++	++	++	+
43	R-F-S	–	+	+	++	++
44	T-S-E	+	+	+	+	++
45	E-S-K	–	+	+	++	+
46	F-R-E	–	+	+	++	+
47	K-I-T	–	+	+	++	+
48	P-N-V	–	+	+	+	+
49	P-R-L	–	+	+	+	+
50	P-T-L	–	–	–	+	+

[a] Mutants, which were screened twice

Table 2 *S. typhimurium* strain MM281 was transformed with p*GEX-Mrs2*(WT) or p*GEX* carrying various *Mrs2* G-M-N mutants (as indicated), serially diluted and replica plated on plates with 0.05 mmol l^{-1} IPTG and different $MnCl_2$ concentrations. The number of ''+'' symbols corresponds to the number of dilution steps exhibiting growth

	Concentration $MnCl_2$ (mmol l^{-1})		
Sequence	0	0.01	0.1
G-M-N	++++	++++	++++
K-R-L	++++	+++	++
R-A-W	++++	+++	++
R-R-T	+++	+++	++
K-A-I	++++	++	+
R-M-V	++++	++	+
R-C-V	++++	+	+
R-F-V	++++	+	+
R-Q-L	++++	+	+
R-V-L	++++	+	+
R-V-H	++	++	+

Table 3 *S. typhimurium* strain MM281 was transformed with p*GEX-Mrs2*(WT) or p*GEX* carrying various *Mrs2* G-M-N mutants (as indicated), serially diluted and replica plated on plates with 0.05 mmol l^{-1} IPTG and different $ZnCl_2$ concentrations. The number of ''+'' symbols corresponds to the number of dilution steps exhibiting growth

	Concentration $ZnCl_2$ (mmol l^{-1})		
Sequence	0	0.01	0.1
G-M-N	++++	++++	+++
K-R-L	++++	+++	++
R-R-T	++++	+++	+++
R-A-W	++++	++	+
K-A-I	++++	++	+
R-C-V	++++	++	++
R-F-V	++++	++	+
R-M-V	++++	++	+
R-Q-L	++++	++	+
R-V-L	++++	++	+
R-V-H	+++	+++	++

wild-type Mrs2p and on the investigated mutants. A feasible explanation might be the disparity between the approaches used by us and by Schindl and coworkers.[21] Co^{2+} concentrations used in the patch-clamp experiments were significantly higher than concentrations used in our experimental setting. Therefore it is not possible to directly compare the results of the growth complementation assay and the patch-clamp experiments of Schindl and colleagues.[21]

The tested concentrations of $MnCl_2$ reduced growth of all mutants, whereas growth of cells harbouring wild-type *MRS2* remained unaffected. The negative effect of Mn^{2+} increased with rising concentrations (Table 2). Furthermore, the growth defect was differently pronounced in the mutants; the top three least affected are characterized by positively charged residues in the first and the second position (K-R-L; R-R-T), while the two most affected mutants carry Arg at the first, and Leu at the third position (R-Q-L; R-V-L) (Table 2).

The effect of Zn^{2+} was similar to Mn^{2+}, however the growth defect of the mutants was slightly less pronounced (Table 3). The three least affected and the two most affected mutants were the same as those identified in the Mn^{2+} assay.

Mg^{2+} influx into isolated mitochondria

In order to directly investigate Mg^{2+} influx into isolated mitochondria of selected mutants we used the Mg^{2+}-sensitive dye mag-fura-2 (Fig. 3). The mutants were selected on the basis of good (R-M-V, R-F-V, R-C-V), medium (R-Q-L) or poor (R-V-H) growth complementation capacity in *S. typhimurium* strain MM281 (Table 1). After addition of Mg^{2+} to a concentration of

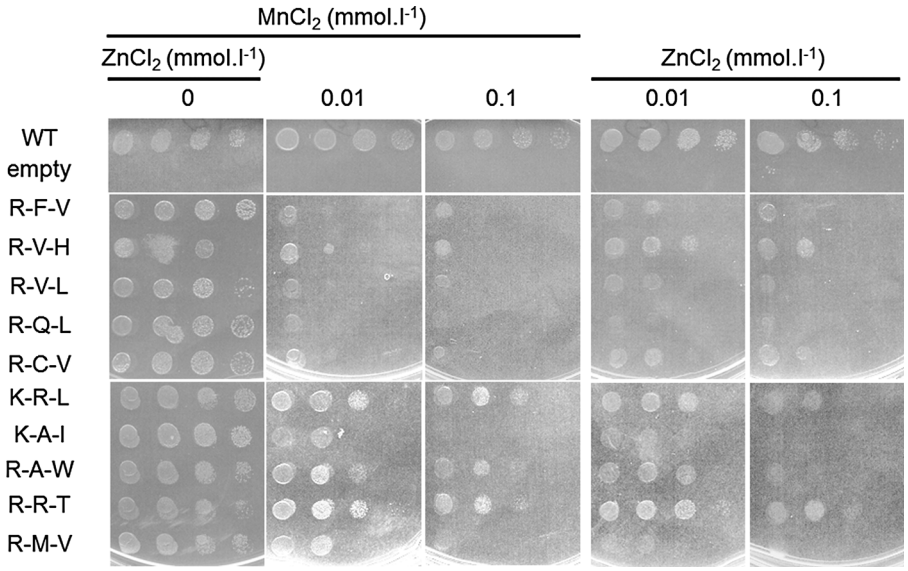

Fig. 2 Influence of Mn^{2+} and Zn^{2+} on growth complementation of selected *MRS2* mutant variants. *S. typhimurium* strain MM281 was transformed with p*GEX-Mrs2*(WT) or p*GEX* carrying various *Mrs2* G-M-N mutants (as indicated), serially diluted and spotted on LB plates supplemented with 0.05 mmol l^{-1} IPTG and different $ZnCl_2$ or $MnCl_2$ concentrations. Plates were incubated at 37 °C for 24 hours.

1 mmol l^{-1} a lack of the characteristic rapid Mg^{2+} influx[9] was observed in most mutants, together with significantly lower steady-state Mg^{2+} levels (Fig. 3). The only exception was the R-Q-L mutant, which lacked rapid Mg^{2+} influx, but reached a mitochondrial Mg^{2+} concentration comparable to the wild-type level during the subsequent 100 seconds. After addition of Mg^{2+} to the final concentration of 3 mmol l^{-1}, we observed Mg^{2+} influx in all mutants; however, it did not reach the final steady-state level of wild-type Mrs2p. The differences between mutants were minimal, with the exception of the R-Q-L mutant, which reached almost wild-type Mg^{2+} levels.

These results indicate that all investigated mutants maintain a certain ability to transport Mg^{2+} in *S. typhimurium* (Table 1) and mutants R-V-H, R-M-V, R-F-V, R-C-V and R-Q-L also in yeast mitochondria (Fig. 3), albeit in both systems the transport activity was significantly decreased compared to the wild type protein.

Discussion

The asparagine residues of the G-M-N motif have been previously proposed to block the entrance of the channel in the closed conformation.[18,22]

In the recent study reported by Pfoh *et al.* it has been suggested that transition of the channel from the closed to the open conformation is mediated by radial and lateral tilts of the protomers that eventually lead to an asymmetric conformation with a bending of the long helix 7 forming the pore.

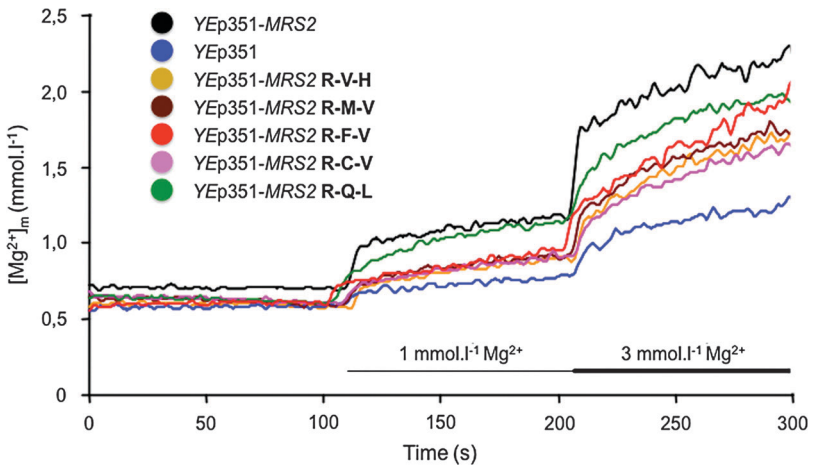

Fig. 3 Representative recordings of $[Mg^{2+}]_m$ in mitochondria carrying WT and mutated Mrs2p upon addition of Mg^{2+} to the measuring buffer. *S. cerevisiae* strain DBY747 *mrs2Δ* was transformed with the indicated plasmids and mitochondria were isolated. The representative recordings show changes in matrix Mg^{2+} concentration ($[Mg^{2+}]_m$) monitored over 300 seconds. $MgCl_2$ was added in step-wise manner to final $[Mg^{2+}]_e$ of 1 and 3 mmol l^{-1}, respectively (as it is indicated).

Moreover, this bending motion was suggested to pull away one of the periplasmic loops at the mouth of the pore rendering the G-M-N motif accessible for ion coordination.[27]

In the model based on the structure of *M. jannaschii* CorA Guskov *et al.* proposed that opening of the channel is mediated by a counterclockwise turn of helix 6 upon loss of Mg^{2+} ions from the intracellular Mg^{2+} binding sites. This movement eventually replaces hydrophobic residues in the pore with polar residues resulting in a hydrophilic ion permeation pathway. Interestingly, this proposed mechanism does not involve any structural rearrangements of the loop.[28]

Taken together, the loop connecting TM1 and TM2 with its G-M-N motif appears to form the initial interaction site for hydrated Mg^{2+} and likely participates in the partial dehydration process of the ion required prior to its entrance into the pore of the channel.[22,27,28] The high conductance of Mrs2p[21] and CorA[22] channels was proposed to be based on a mechanism which involves electrostatic interactions of the loop residues with the hydration shell of Mg^{2+} and not with the ion itself.[22] This notion is supported by the two recently published structures in which a partially hydrated Mg^{2+} ion was observed in the loop region at the level of the G-M-N motif with a distance of 4 Å from the coordinating amino acid residues.[27,28]

Our study on the G-M-N motif identified viable triple mutants hosting a positively charged residue primarily on the first but also on the third position. At a first glance, the presence of positively charged residues in functional mutants might seem counterintuitive as these mutations could in fact hinder the transport of Mg^{2+} ions by electrostatic repulsion. However, it is structurally unfeasible for all three amino acid residues of the motif to be in direct contact with the ion.[26–28] It is plausible to envisage that these residues form a structural motif critical for ion uptake, which can be partially accomplished by different amino acid combinations, eventually leading to a functionally equivalent structure. Furthermore, it is possible to hypothesize that backbone interactions with the ion cargo are more important than side-chain interactions, which would explain the broad variation of amino acids capable of sustaining selectivity and transport.

In order to assess the impact of mutations in the G-M-N motif on the selectivity of Mrs2p, we performed growth complementation assays on plates supplemented with different cations (Ca^{2+}, Co^{2+}, Mn^{2+} and Zn^{2+}). Our results show that mutations in the G-M-N motif lead to reduced growth of the cells in the presence of Mn^{2+} and Zn^{2+} while Ca^{2+} and Co^{2+} did not influence their viability. This can take place *via* two possible competitive mechanisms: (1) Mn^{2+} and Zn^{2+} are transported through the pore and the growth defect is caused by Mn^{2+}/Zn^{2+} intoxication, or (2) Mn^{2+} and Zn^{2+} ions are trapped and block the channel for Mg^{2+} transport causing in this way the growth defect by Mg^{2+} deficiency. Further studies are needed to clarify the effects of an alteration of the G-M-N sequence on the structural characteristics in this region of the channel.

For CorA[25,26] and Mrs2p[9] proteins it has been shown that even very conservative mutations in this sequence are not tolerated. However, we were able to identify viable mutants with amino acids greatly differing from G-M-N. This suggests that the individual amino acids of this motif are not critical but the structural properties generated collectively by this motif. This can be partially accomplished by amino acid combinations of completely different individual properties. It seems that G-M-N is the best combination nature found to ensure a high selectivity combined with a high transport capacity.

Materials and methods

Bacterial cells, growth media and genetic procedures

Escherichia coli DH10B: F-; endA1; recA1; galE15; galK16; nupG; rpsL; ΔlacX74; Φ80lacZΔM15; araD139 Δ(ara,leu)7697; mcrA Δ(mrr-hsdRMS-mcrBC) λ; *Salmonella enterica* serovar *Typhimurium* transmitter strain LB5010: metA22; metE551; ilv-452; leu-3121; trpC2; xyl-404; galE856; hsdL6; hsdSA29; hsdSB121; rpsL120; H1-b H2-e n x flaA66 nml (–) Fel-2(–); Mg^{2+} dependent *Salmonella enterica* serovar *Typhimurium* strain MM281: DEL485(leuBCD); mgtB::MudJ; mgtA21::MudJ; corA45::mudJ; zjh1628::Tn10(cam); Cam^R, Kan^R (it lacks all major magnesium transport systems and therefore requires medium containing Mg^{2+} concentrations in the millimolar range); *Salmonella enterica* serovar *Typhimurium* strain MM1927: DEL485(leuBCD); mgtB::MudJ; mgtA21::MudJ; corA45::mudJ; zjh1628::Tn10(cam); Cam^R, Kan^R, pALTER-CorA (Amp^R).

Strains were grown in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) with ampicillin (100 μg ml^{-1}). MM281 required addition of 10 mmol l^{-1} $MgCl_2$. LB plates contained 2% Difco Agar Noble minimizing possible Mg contamination.

Yeast cells, growth media and genetic procedures

The *S. cerevisiae* strain DBY747 *mrs2Δ* deletion strain (DBY mrs2-1, short) has been described previously.[12–14] Yeast cells were grown in rich medium (YPD; 1% yeast extract, 2% peptone and 2% glucose).[11]

Plasmid constructs

The construct YEp351-MRS2-HA was described previously by Bui and coworkers.[17] For cloning of *MRS2* into the vector pGEX-3X with an IPTG-inducible promoter the primers M2GEXfw: 5′-CGCGGATCCCCAATCGGCGTCTCCTGG-3′ and MRS2HiXrev: 5′-TGCTCTAGATCAATGGTGATGGTGATGG-3′ were used. The resulting PCR fragment was cloned into the vector *via* BamHI and XbaI restriction sites.

Random PCR mutagenesis

In order to introduce various amino acid substitutions in Mrs2p, overlap extension PCR according to Pogulis and coworkers[30] was used. No additional mutations were found by sequencing. Random mutagenesis of the G-M-N motif of *MRS2* was performed with the mutagenic forward primer 5′-GCATCTGTTCTGCCGGCGTTCTATN NNNNNNNNTTAAAGAATTTCATCGAGGAGAGTG-3′ and the reverse primer 5′-CACTCTCCTCGATGAAATTCTTTAANNNNNNNNNATAG AACGCCGGCAGAACAGATGC-3′ according to standard protocols. PCR products were digested with XhoI and EcoRI and cloned into an XhoI and EcoRI digested pGEX-*MRS2* construct. For transformation into DH10B a standard calcium chloride method was used.

Correctly ligated constructs were identified by deletion of the BsmI restriction site of *MRS2*, resulting in a silent mutation from an adenine to a guanine.

Identification of tolerated substitutions

A total of 45 600 constructs were pooled and transformed into *S. typhimurium* strain LB5010. A total of 46 848 constructs were pooled and transformed into *S. typhimurium* strain MM281, plated on LB plates supplemented with 10 mmol l^{-1} $MgCl_2$ and replica plated on LB plates containing 0.05 mmol l^{-1} IPTG to induce protein expression. Expression of the mutant Mrs2p variants was controlled by Western blot to avoid misinterpretations due to strongly differing expression levels. Forty nine mutants able to grow on this medium were sequenced. No additional mutations were found by sequencing.

Serial dilutions

For serial dilutions on plates, cells were grown in liquid LB medium containing 10 mmol l^{-1} $MgCl_2$ at 37 °C overnight, washed twice with LB medium, adjusted to an A_{600} of 1 and diluted to an A_{600} 0.1, 0.01 and 0.001. Serial dilutions were spotted onto LB medium plates containing different concentrations of $MgCl_2$, IPTG, $MnCl_2$ or $ZnCl_2$ and incubated for 24 h.

Isolation of mitochondria and measurement of changes in the intramitochondrial Mg^{2+} concentrations by mag-fura 2 assisted spectrofluorimetry

Isolation of mitochondria by differential centrifugation and ratiometric determination of intramitochondrial Mg^{2+} concentrations ($[Mg^{2+}]_m$) dependent on various external concentrations ($[Mg^{2+}]_e$) was performed as described previously.[9]

Conclusions

In summary we conclude that the G-M-N motif, despite its high degree of conservation, can be functionally replaced by certain combination(s) of amino acid residues. Most frequently a positively charged residue in the first and a hydrophobic residue in the third position were found in functional mutants. Our studies suggest that the G-M-N motif plays a role in ion selectivity, being therefore part of the selectivity filter together with the flanking negatively charged loop, at the entrance of the Mrs2p channel. The eminent involvement of the G-M-N motif in ion selectivity might be the molecular basis for its universal conservation throughout the phyla.

Authors' contributions

GS, SS and MBK conducted experiments; RS, GS and KDC designed experiments; RS, GS, SS, MBK, MK, OC and KDC evaluated data; MK, GS, SS, OC and KDC wrote the manuscript.

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