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The yeast mitochondrial carrier proteins Mrs3p/Mrs4p mediate iron transport across the inner mitochondrial membrane

Elisabeth M. Froschauer, Rudolf J. Schweyen [1], Gerlinde Wiesenberger *[,2]

Max F. Perutz Laboratories, Department of Genetics, University of Vienna, Dr. Bohrgasse 9, A-1030 Vienna, Austria

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ABSTRACT

The yeast proteins Mrs3p and Mrs4p are two closely related members of the mitochondrial carrier family (MCF), which had previously been implicated in mitochondrial Fe^{2+} homeostasis. A vertebrate Mrs3/4 homologue named mitoferrin was shown to be essential for erythroid iron utilization and proposed to function as an essential mitochondrial iron importer. Indirect reporter assays in isolated yeast mitochondria indicated that the Mrs3/4 proteins are involved in mitochondrial Fe^{2+} utilization or transport under iron-limiting conditions. To have a more direct test for Mrs3/4p mediated iron uptake into mitochondria we studied iron (II) transport across yeast inner mitochondrial membrane vesicles (SMPs) using the iron-sensitive fluorophore PhenGreen SK (PGSK). Wild-type SMPs showed rapid uptake of Fe^{2+} which was driven by the external Fe^{2+} concentration and stimulated by acidic pH. SMPs from the double deletion strain mrs3/4Δ failed to show this rapid Fe^{2+} uptake, while SMPs from cells overproducing Mrs3/4p exhibited increased Fe^{2+} uptake rates. Cu^{2+} was transported at similar rates as Fe^{2+}, while other divalent cations, such as Zn^{2+} and Cd^{2+} apparently did not serve as substrates for the Mrs3/4p transporters. We conclude that the carrier proteins Mrs3p and Mrs4p transport Fe^{2+} across the inner mitochondrial membrane. Their activity is dependent on the pH gradient and it is stimulated by iron shortage.

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1. Introduction

The mitochondrial carrier family (MCF) is a large group of structurally related membrane proteins which are conserved in eukaryotes. Members of the mitochondrial carrier family exhibit a tripartite structure; each of the three parts consists of about 100 amino acids sharing sequence similarity with the other modules. Most members of the mitochondrial carrier family are located in the inner mitochondrial membrane and transport various solutes between the cytosol and mitochondria. Substrates of these proteins vary in size and structure ranging from protons to large molecules such as ATP and ADP. The roles of most of the 35 members in yeast have been established by expressing the carriers in *E. coli* and reconstituting the purified proteins in liposome vesicles (reviewed in [1]).

While most mitochondrial carrier proteins studied so far transport metabolites such as amino acids, keto acids, nucleotides, only a few members of this family have been implicated in mitochondrial ion homeostasis. Several lines of evidence link the closely related proteins Mrs3p and Mrs4p with iron transport into mitochondria [2–5]. Additionally, the mitochondrial carrier proteins Mtm1p and Ggc1p/Yhm1p are thought to play a direct or indirect role in mitochondrial iron homeostasis [6–9]. Recently, it was demonstrated, that uncoupling proteins 2 and 3 (Ucp2 and Ucp3) are essential for mitochondrial Ca^{2+} uptake [10].

The crystal structures of two members of the mitochondrial carrier family have been solved to date [11,12]. Robinson and Kunji identified common residues responsible for substrate recognition in mitochondrial carrier proteins [13,14]. Sequence alignment and clustering of the carriers based on their substrates revealed 3 regions in trans-membrane helices 2, 4 and 6, which are conserved in carriers with certain substrate specificity (contact points I, II and III). Interestingly, the yeast proteins Mrs3p and Mrs4p share a novel MN motif at contact point II, suggesting that they transport neither nucleotides nor keto acids nor amino acids and form a distinct subfamily within the MCF [13].

MRS4 is co-regulated with the iron regulon genes [2,15], and changes in the *MRS3/4* expression lead to up-regulation of plasma-membrane iron transporters and increased cellular uptake of iron [3,16]. Using assays for bio-available iron in yeast mitochondria several groups presented data strongly suggesting a role of Mrs3p and Mrs4p in the transport of iron into mitochondria [2–5].

Deletion of either *MRS3* or *MRS4* results in no apparent phenotype. Simultaneous deletion of both genes, however, causes pleiotropic

* Corresponding author. Muthgasse 18, A-1190 Vienna, Austria. Tel.: +43 1 36006 6381.
E-mail address: gerlinde.wiesenberger@boku.ac.at (G. Wiesenberger).

[1] This paper is dedicated to the memory of Rudolf Schweyen who tragically died during the reviewing process of this manuscript.

[2] Current address: Institute of Applied Genetics and Cell Biology, BOKU-University of Natural Resources and Applied Life Sciences, A-1190 Vienna, Austria.

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phenotypes like poor growth at elevated temperatures, altered heavy metal resistance and tolerance, the most significant being resistance to Co^{2+}, hypersensitivity to Cd^{2+} and a growth defect on low iron media [2,3,16]. The latter phenotype is consistent with the observation that *MRS3* and *MRS4* are involved in uptake of Fe^{2+} into mitochondria only under iron-limiting conditions [3].

Genes encoding homologues of Mrs3/4 have been identified in many higher eukaryotes. Evidence for their involvement in iron homeostasis comes from the analysis of a zebrafish mutant with a defect in the Mrs4 homologue mitoferrin (mfrn). This mutant shows profound hypochromic anaemia and erythroid maturation arrest suggesting defects in mitochondrial iron uptake [17].

In order to test if the Mrs3/4 proteins mediate iron transport we have measured the Fe^{2+} flux across the mitochondrial membrane by using an assay involving the fluorescent dye PhenGreen SK (PGSK) entrapped in inner membrane vesicles [18,19]. Fluorescence of PGSK is quenched by chelatable (free) Fe^{2+} and other divalent ions in a concentration dependent manner. This fluorophore is selectively sensitive to Fe^{2+} over Fe^{3+} [20,21]. The use of membrane vesicles, e.g. consisting of inner mitochondrial membranes (submitochondrial particles, SMPs) with entrapped fluorescent dyes allowed us to modify at will the external and internal ion concentrations and to observe time-dependent changes in fluorescence reflecting changes in internal concentrations due to diffusion or the action of transport proteins [22].

2. Materials and methods

2.1. Yeast strains, plasmids and media

DBY747 (ATCC no. 204659) was used as a wild-type strain. Disruption of *MRS3* and *MRS4* was performed in DBY747 by stepwise deletion of both coding regions using the short flanking homology gene disruption method and *loxP* flanked selection markers [23]. After verification of the deletions by diagnostic PCR the selection markers were removed using a plasmid containing the *CRE*-recombinase. In the resulting strain GW403 (mrs3/4Δ; MATa *his3-Δ1 leu2-3 leu2-112 ura3-52 trp1-289 mrs3Δ::loxP mrs4Δ::loxP*) the open reading frames of *MRS3* and *MRS4* are replaced by the *loxP* sequence. For simultaneous overexpression of *MRS3* and *MRS4* (MRS3/4n), DBY747 was transformed with plasmids pGW821 (*MRS4*; *URA3*) and pGW822 (*MRS3*; *LEU2*), which express the open reading frames of the respective genes under control of the *ADC1* promoter [3]. DBY747 transformed with the empty plasmids pVT-102-U [24] and pAAH5 [25] served as control.

For isolation of mitochondria prior to SMP preparation DBY747 and GW403 were grown in YPD (1% yeast extract, 2% peptone, 2% dextrose) to stationary phase, while transformed strains were grown in synthetic complete media lacking uracil and leucine [26]. Iron-free media were prepared using YNB without iron purchased from ForMedium™ (Norfolk, UK).

2.2. Reagents

All reagents and buffers used for preparation of SMPs and for iron flux measurements were prepared in ion free water (Fluka). PhenGreen SK was purchased from Molecular Probes (Invitrogen), diluted to a stock concentration of 10 mM and used at a final concentration of 50 μM. Iron (II) containing stock solutions (1 mM) containing 0.1 M acetic acid and 10 mM ascorbate were freshly prepared each experimental day. Pyrithione (Sigma) was diluted to a concentration of 10 mM in DMSO and used at a final concentration of 20 μM.

2.3. Preparation of sub-mitochondrial particles (SMPs), loading with PhenGreen SK and fluorescence measurements

The preparation of SMPs was performed essentially as described previously [22]. The SMP pellet was resuspended in sucrose buffer (250 mM sucrose, 10 mM TrisCl pH 7.4) containing 50 μM of PhenGreen SK. To entrap the dye within the SMPs, this suspension was pulse-sonified on ice three times for 60 s with 80% intensity in a Bandelin sonicator UW70/GM70. SMPs were collected by ultracentrifugation (60,000 *g*, 10 min, 4 °C), washed once and used immediately at a final concentration of 0.025 mg protein/ml.

Fluorescence intensities were recorded with a Perkin Elmer LS-55 Fluorimeter using the TimeDrive program where the emission was set at a wavelength of 520 nm after an excitation at 506 nm. The measurements were done within 1 h after preparation of loaded SMPs in 3-ml cuvettes containing 2 ml of SMP suspension with stirring at 25 °C. Unless otherwise stated, pH was set at 7.4 with 10 mM TrisCl.

Leakage of PGSK from SMPs was regularly controlled by measuring fluorescence in the external buffer after removal of SMPs by centrifugation. In control experiments at the end of each series of measurements the metal ionophore pyrithione (20 μM) was added to the samples and incubated for 5 min prior to the addition of Fe^{2+} to observe maximal quenching of PGSK with a given Fe^{2+} concentration.

Fluorescence of PhenGreen SK was not affected by DMSO, acetic acid, ascorbate or pyrithione.

2.4. Standard curve for quenching of PGSK

The curves shown in the figures represent the fluorescence intensities collected at an emission wavelength of 520 nm after an excitation at 506 nm. To calculate total concentrations, calibration curves were produced for every ion used for flux studies. PGSK (1 μM) was resuspended in 1 ml sucrose buffer and the fluorescence at ion concentration = 0 was determined (F_0). The ion concentration was increased stepwise up to 15 μM and the different fluorescence intensities were used to determine the (F_0/F)–1 ratio which was plotted against the corresponding ion concentration.

Each experiment was repeated at least three times with different SMP preparations. Ion concentrations given in the text are means plus standard deviations of the least three different concentration calculations ($n>3$).

3. Results

3.1. PGSK assays for the detection of Fe^{2+} uptake into submitochondrial vesicles

Based on analysis of iron-utilizing processes the mitochondrial carrier proteins Mrs3p and Mrs4p have been suggested to be involved in mitochondrial iron uptake [3,5]. In order to directly assess the contribution of Mrs3p and Mrs4p to Fe^{2+} transport across the inner mitochondrial membrane we employed the membrane-impermeable fluorescent dye PhenGreen SK (PGSK) entrapped in yeast submitochondrial particles (SMPs). A similar assay has been used previously to measure Fe^{2+} uptake into vesicles generated from the inner envelope membrane of pea chloroplasts [18,19]. SMPs have been shown to be a suitable tool to measure ion fluxes across the mitochondrial membrane: ion leakage was found to be low and driving forces for the transport of ions by proteins could be generated at will by controlling internal and external ion concentrations [22]. Fluorescence of PGSK is quenched by chelatable iron as well as by other metal ions. This poor ion-selectivity is of minor relevance in the assays shown here because PGSK-containing SMPs were kept in metal ion free buffers and were challenged with a single metal ion only.

Mitochondria were isolated from wild-type cells (WT) and from cells deleted for both genes (mrs3/4Δ), SMPs (submitochondrial particles) were prepared and loaded with 5 μM of PGSK as described in Materials and methods. SMPs contained identical, nominally Fe^{2+}-free sucrose buffers (TrisCl pH 7.4) inside and outside. When monitored at 520 nm with excitation at 506 nm, fluorescence of PGSK-loaded SMPs (cf. Methods) was threefold higher than autofluorescence of unloaded SMPs

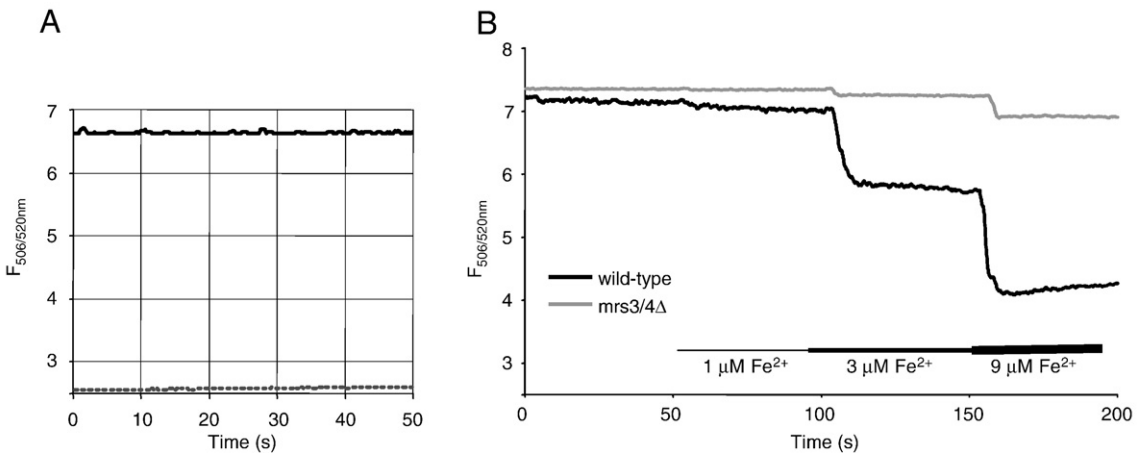

Fig. 1. SMPs of wild-type (DBY747) and *mrs3/4Δ* (GW403) react differently to stepwise increase of outside Fe(II) concentration. SMPs loaded with PGSK were prepared from mitochondria isolated from strains DBY747 (wt) and GW403 (*mrs3/4Δ*) as described in Materials and methods. (A) Fluorescence of PGSK-loaded wild-type SMPs (black solid line) versus auto fluorescence of unloaded SMPs (grey dotted line). (B) $FeSO_4$ was added to PGSK-loaded SMPs to the final concentrations indicated in the figure and quenching of PGSK fluorescence was recorded. Wild-type, black line; *mrs3/4Δ*, grey line.

(Fig. 1A). As shown in Fig. 1B fluorescence of PGSK-loaded wild-type SMPs showed a rapid decrease in response to stepwise addition of $FeSO_4$ to the reaction buffer ($[Fe^{2+}]_o$). This effect was complete within a few seconds after Fe^{2+} addition and fluorescence stayed roughly constant thereafter. In contrast, identically treated PGSK-loaded mutant *mrs3/4Δ* SMPs showed only a marginal decrease in fluorescence.

When SMPs were pre-incubated with pyrithione, a metal ionophore [27,28] wild-type SMPs and mrs3/4Δ SMPs exhibited similar capacities for Fe^{2+} uptake, while untreated mutant mrs3/4Δ SMPs failed to take up iron (Fig. 2A). Using calibration curves (see Material and methods) and the fluorescence quenching data of Fig. 2A intravesical Fe^{2+} concentrations ($[Fe^{2+}]_i$) were calculated (Fig. 2B). Based on these data we assume, that SMPs lacking the Mrs3 and Mrs4 proteins have very little leakage for Fe^{2+} and that the fluorescent dye stays entrapped inside the SMPs.

3.2. Fe^{2+} flux is dependent on the expression of MRS3 and MRS4

We next tested whether Fe(II) flux into SMPs correlates with the expression levels of *MRS3* and *MRS4*. As shown in Fig. 3A addition of Fe(II) to SMPs from cells overexpressing *MRS3* and *MRS4* resulted in increased quenching of PGSK fluorescence. Upon addition of 5 μM Fe^{2+} to SMPs prepared from a strain over-expressing *MRS3* and *MRS4*, the steady-state concentration of Fe(II) inside the SMPs was 4.18 ± 0.06 μM, which is substantially higher than the 3.48 ± 0.26 μM measured in wild-type SMPs (Fig. 3B). As obvious from Panel A, iron uptake into SMPs from *MRS3/4* overexpressing cells was much faster than that into wild-type SMPs. The uptake-rate was calculated to be 0.52 ± 0.09 μM/s/mg protein, which is about 10% higher than the uptake rate of 0.43 ± 0.05 μM/s/mg in SMPs originating from wild-type cells (Fig. 3C). In summary, SMPs lacking Mrs3/4p fail to exhibit $[Fe^{2+}]_o$-driven Fe^{2+} uptake and SMPs with excess Mrs3/4p exhibit increased capacities of this Fe^{2+} uptake. Accordingly, we conclude that mitochondrial carriers Mrs3/4p transport Fe^{2+} across the inner mitochondrial membrane.

3.3. SMPs from iron-starved yeast cells have increased Fe^{2+} flux

Growth of yeast cells under iron-limitation results in increased *MRS4* expression (GW unpublished data) and Mrs3/4p dependent bioavailability of iron was only seen in mitochondria from iron-starved yeast cells [3]. To determine whether iron starvation during

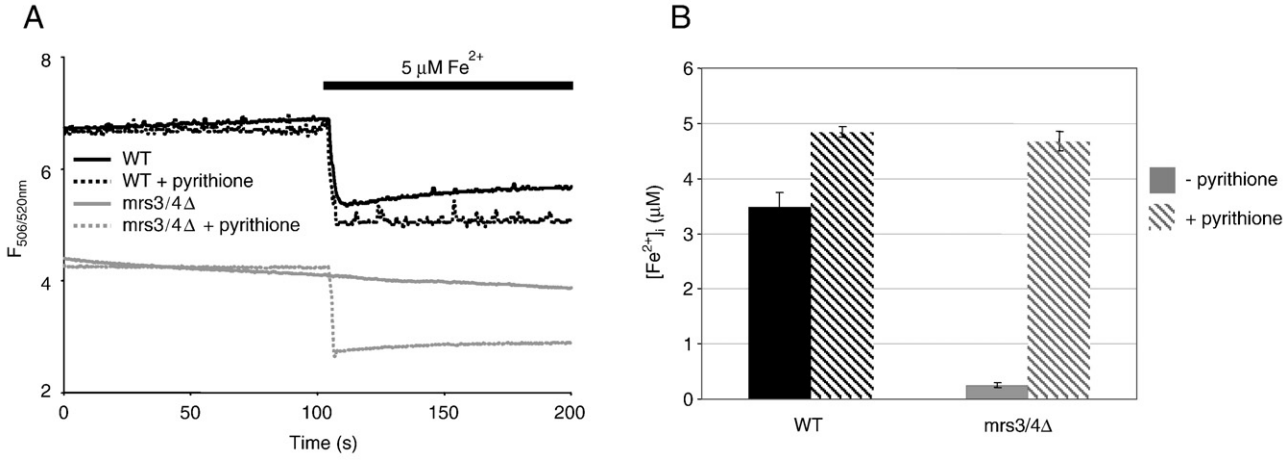

Fig. 2. SMPs are tight and $[Fe^{2+}]_o$ driven Fe^{2+} flux can be artificially induced using the ionophore pyrithione. (A) SMPs prepared from wild-type (DBY747, black lines) and *mrs3/4Δ* (GW403, grey lines) mitochondria were loaded with PGSK and resuspended in nominally Fe^{2+}-free sucrose buffer. $FeSO_4$ was added to the SMPs to a final concentration of 5 μM and quenching was recorded. Where indicated (dotted lines) SMPs were pre-incubated with pyrithione as described in Materials and methods. (B) Fe^{2+} concentrations within SMPs ($[Fe^{2+}]_i$) were calculated from five (−pyrithione) or eight (+pyrithione) independent measurements, respectively, as described in Materials and methods.

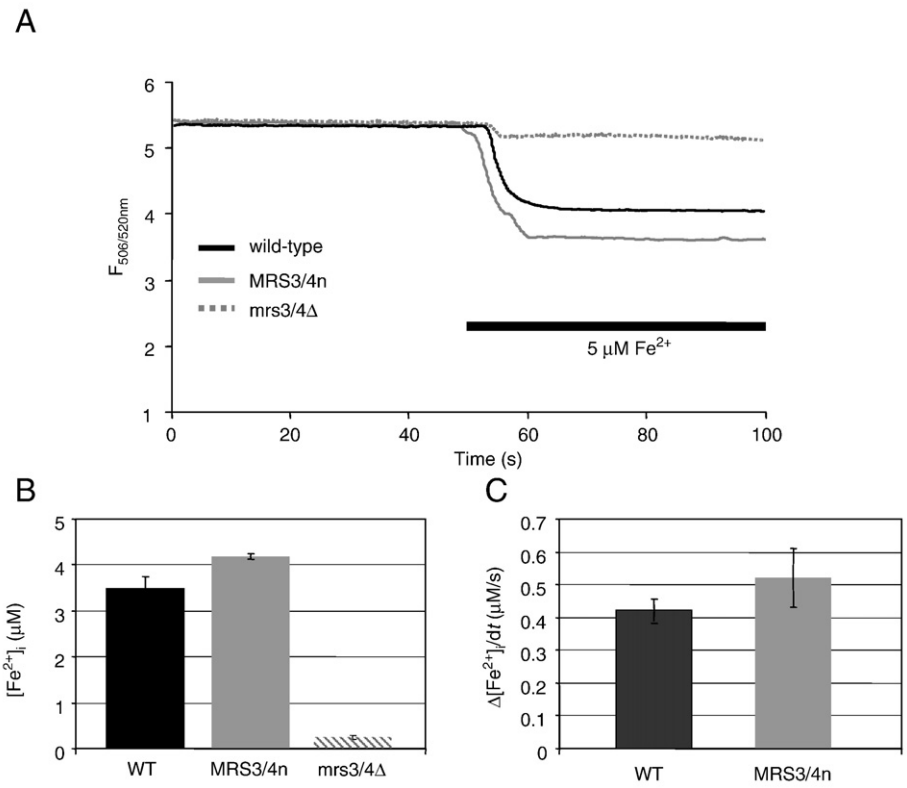

Fig. 3. Fe^{2+} influx is dependent on the expression levels of *MRS3* and *MRS4*. (A) SMPs were prepared from wild-type (DBY747 transformed with plasmids pAAH5 and pVT-103U; black line), MRS3/4n (DBY747 transformed with pGW822 and pGW821, grey line) and *mrs3/4Δ* (GW403; grey dotted line) mitochondria, loaded with PGSK, resuspended in nominally Fe^{2+}-free sucrose buffer and challenged with 5 μM $FeSO_4$. Quenching of PGSK fluorescence was recorded. (B) Fe^{2+} concentrations within SMPs ($[Fe^{2+}]_i$) were calculated from five independent measurements as described in Materials and methods. (C) Fe^{2+} uptake rates were determined from five fluorescence recordings for each condition using Fe^{2+} concentrations determined as described in Materials and methods.

growth of the cells influences the iron transport capacities of SMPs we compared Fe^{2+} uptake by SMPs from wild-type and *MRS3/4* overexpressing cells grown under iron-replete and iron-deplete conditions (Fig. 4). Upon addition of 5 μM Fe^{2+} to SMPs from iron-starved cells both, WT and MRS3/4n SMPs, showed increased steady-state $[Fe^{2+}]_i$ (4.92 μM ± 0.06 and 4.90 μM ± 0.03, respectively) corresponding to a 42% increase in wild-type SMPs and a 17% increase in MRS3/4n SMPs compared to SMPs from cells grown in standard medium (Fig. 4B). No Fe^{2+} flux was detected in SMPs from the *mrs3/4Δ* strain regardless of the growth conditions (data not shown). Iron influx ($\Delta[Fe^{2+}]_i$/s/mg protein) was also higher in the SMPs from iron-starved cells. In wild-type SMPs the influx rate was 1.23 ± 0.17 μmole Fe^{2+}/s/mg protein, while it was 1.63 ± 0.12 μmole Fe^{2+}/s/mg protein in MRS3/4n SMPs, representing a 3-fold increase in both cases, when compared to the uptake rates in SMPs from cells grown under iron-replete conditions (Fig. 4C).

3.4. Driving force for Mrs3/4p mediated Fe(II) transport

The inner mitochondrial membrane separates two compartments differing in pH, the matrix (alkaline) and the intermembrane space (acidic). The inward directed pH gradient, formed by the respiratory chain, contributes the bulk of the mitochondrial membrane potential ($\Delta\psi$). In all experiments described above, the milieu inside and outside of the SMPs was identical, except for the presence of PGSK inside and iron salts added to the external buffer, with a pH of 7.4 inside and outside. Accordingly, the inside directed Fe^{2+} gradient appears to be the driving force for this transport.

To determine, whether pH influences the Fe(II) flux across the inner mitochondrial membrane, we performed experiments where the internal pH of wild-type SMPs was set to 7.0 and the external pH varied between 6.0 and 8.0. As shown in Fig. 5A $[Fe]_o$ dependent quenching of PGSK was higher when the outside pH was below pH 7.0, while no quenching was observed at pH_o of 8.0. We then determined the steady-state levels of $[Fe^{2+}]_i$ after addition of 5 μM Fe^{2+} to the external buffer in a more detailed study (Fig. 5B): the internal steady-state levels of Fe^{2+} were around 3.20 μM, when the external pH was kept between 6.8 and 7.0, while more iron (II) accumulated in SMPs which were incubated in lower pH_o (pH 6.0–6.8). On the other hand Fe^{2+} uptake was reduced, when the external pH exceeded 7.2 and became negligible at $[pH]_o$ greater than 7.8. At this pH $[Fe^{2+}]_i$ was less than 10% of the maximal value seen with pH_o of 6.0. Similar data were obtained with SMPs from cells overexpressing *MRS3* and *MRS4* (data not shown). It should be noted that in previous experiments, where pH_i and pH_o were set to 7.4, the internal Fe^{2+} concentration was 3.48 ± 0.26 μM (see Figs. 2 and 3), while we see internal iron concentrations that are considerably lower (2.54 ± 0.14 μM) when pH_i is 7.0 and pH_o is 7.4 (Fig. 5B), indicating that reduced solubility of iron at higher pH is not causing the observed decrease of Fe^{2+} import. We conclude from this, that the inside-directed pH gradient and/or the $\Delta\psi$ resulting thereof strongly stimulate Fe^{2+} uptake by Mrs3/4p into SMPs.

3.5. Ion selectivity of Mrs3/4p

Quenching of PGSK fluorescence can be observed not only with Fe(II) ions but also with other divalent ions, notably for the physiologically relevant ions Cu^{2+}, Mn^{2+}, Co^{2+} and Zn^{2+}, but not for Ca^{2+} or Mg^{2+} (data not shown). Also, while addition of Mn^{2+} or Co^{2+} does quench PGSK fluorescence, quenching does not occur in a concentration dependent manner, thus we were not able to use these ions in uptake assays. Addition of $ZnCl_2$ added to wild-type SMPs at final concentrations of 0.75 to 50 μM did not elicit significant quenching of PGSK, indicating that SMPs lack transport systems for Zn^{2+} working

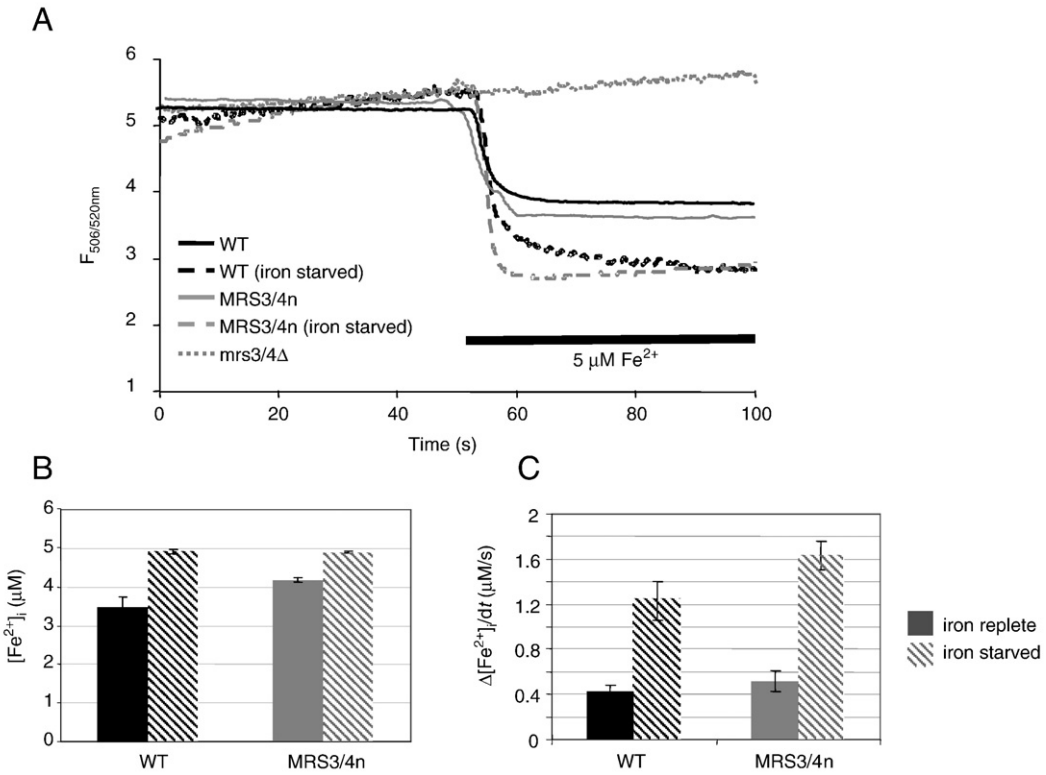

Fig. 4. Growth under iron-limiting conditions increases the Fe^{2+} influx. (A) quenching of PGSK fluorescence in SMPs from wild-type (black lines), *MRS3*/*MRS4* over-expressing (grey lines) and *mrs3*/*4Δ* cells (grey dotted lines) which were either grown under standard (iron-replete medium, solid lines) or under iron-limiting (iron-free medium, dashed lines) conditions. (B) Fe^{2+} concentrations within SMPs ($[Fe^{2+}]_i$) were calculated for five independent samples (iron starvation) or three independent samples (iron-replete conditions), respectively as described in Materials and methods. (C) Fe^{2+} uptake rates were determined from all fluorescence recordings using Fe^{2+} concentrations determined as described in Materials and methods.

under the conditions applied here (Fig. 6 and data not shown). In contrast, Cu^{2+} seems to be transported by the Mrs3/4 proteins. As shown in Fig. 6, SMPs from wild-type mitochondria accumulate Cu(II) to a concentration of about 2.01 ± 0.33 μM upon addition of 5 μM $CuSO_4$ to the external buffer, while very little Cu(II) accumulation was observed in SMPs from *mrs3*/*4Δ* mitochondria. Accordingly, we conclude that Mrs3/4p transport Cu^{2+} as well as Fe^{2+}, but Zn^{2+} is not transported under these conditions. With equal concentrations

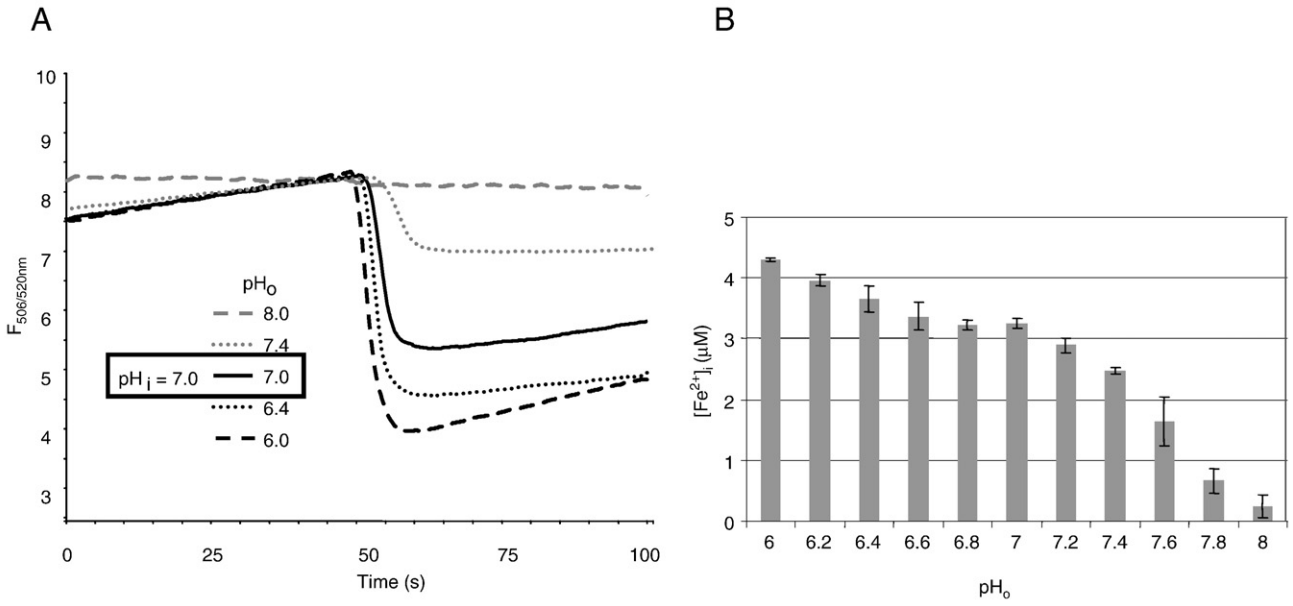

Fig. 5. An inward directed ΔpH enhances Fe^{2+} import into wild-type SMPs. (A) Wild-type SMPs prepared in sucrose buffer pH 7.4 were resuspended in buffers with different pH ranging from 6 to 8.4. Dashed grey line, pH_o 8.4; dotted grey line, pH_o 8.0; solid black line, pH_o 7,4; dotted black line, pH_o 7.0; dashed black line, pH_o 6.4. Quenching curves of selected samples are shown. (B) Quantification of the experiment described in (A) using more pH points. All measurements were performed at least three times.

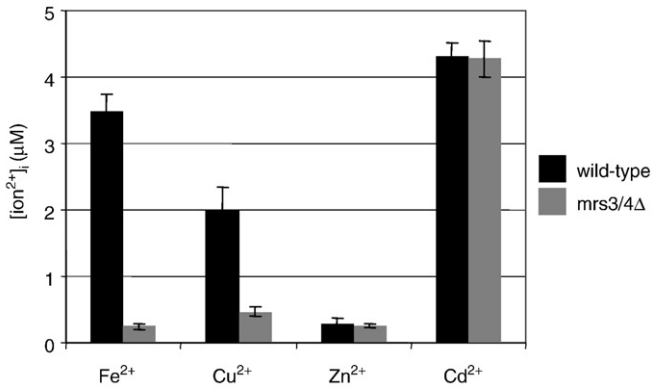

Fig. 6. Ion selectivity of Mrs3p/4p. SMPs prepared from wild-type (DBY747, black bars) and *mrs3/4Δ* (GW403, grey bars) mitochondria were loaded with PGSK and resuspended in nominally ion-free sucrose buffer. Chloride salts of Fe^{2+}, Cu^{2+}, Zn^{2+} or Cd^{2+} were added to the SMPs to a final concentration of 5 μM and quenching was recorded. $[I^+]_i$ was determined in wild-type and mutant SMPs as described in Materials and methods. Each experiment was performed in triplicate.

applied outside (5 μM Fe^{2+} or Cu^{2+}, respectively) the $[Cu^{2+}]$ reached inside the SMPs was roughly two-fold lower than $[Fe^{2+}]$ while the copper transport rate of 0.42 ± 0.24 μmol Fe^{2+}/s/mg protein, was in a similar range as that for iron. Addition of Cd^{2+}, which is also able to quench PGSK fluorescence efficiently, strongly reduced fluorescence of PGSK both in wild-type SMPs and in *mrs3/4Δ* mutant SMPs (Fig. 6). Thus we assume that Cd^{2+} can cross the inner mitochondrial membrane independent of the presence of Mrs3/4 proteins.

4. Discussion

Mrs3p and Mrs4p, two members of the MCF family, have previously been shown to be involved in mitochondrial Fe^{2+} homeostasis, putatively by contributing to Fe^{2+} transport across the inner mitochondrial membrane [2,3,5]. Mutation of one Mrs3/4 homologue (termed mitoferrin 1) causes hypochromic anemia and erythroid maturation arrest in zebrafish [17], suggesting an essential role of mitochondrial iron homeostasis in vertebrate erythropoesis.

Here we provide the first direct evidence for Mrs3/4p-mediated Fe^{2+} transport across the yeast inner mitochondrial membrane, driven by both the concentration gradient of this ion and ΔpH. Moreover, we show that Mrs3/4p can also transport Cu^{2+}, but not Zn^{2+} and Cd^{2+}. Thus, Mrs3p and Mrs4p constitute the first known metal ion transporters among the 35 yeast MCF proteins.

The activity of transporters can be readily studied using membrane vesicles because milieus inside and outside the vesicles as well as concentrations of factors stimulating or inhibiting transport of a substrate can be varied at will. Here we made use of submitochondrial particles (SMPs) prepared from yeast mitoplasts (with disrupted outer membranes) loaded with a fluorescent dye (PhenGreen SK) to monitor intravesicular ion concentrations. In line with previous observations [22], we find little leakage of ions, protons or entrapped dyes across SMP membranes. Low ion-selectivity of PGSK [29] causes no problems in the assays performed here because PGSK-loaded SMPs are challenged with only one metal ion per assay, with no competing cations present in the buffer.

Addition of Fe^{2+} to SMPs originating from cells expressing Mrs3/4p resulted in rapid quenching of PGSK fluorescence indicating transport of the ion into the vesicles, while SMPs lacking Mrs3/4p failed to take up iron. Moreover, SMPs with increased doses of Mrs3/4p exhibited significantly increased Fe^{2+} uptake. Accordingly, we conclude that the MCF proteins Mrs3/4p mediate Fe^{2+} transport across the inner mitochondrial membrane. The transport substrate is likely to be Fe^{2+} ion in its free form but we cannot exclude that acetate or ascorbate present in the buffers play a role in Fe^{2+} uptake. Ligands which *in vivo* may bind iron in the cytoplasm are unlikely to be cofractionated with SMPs in concentrations equivalent to the added Fe^{2+}.

With equal buffers inside and outside the SMPs, i.e. in the absence of a pH gradient or membrane potential, SMPs showed a strong and rapid uptake of Fe^{2+} upon addition of 5 μM iron sulphate to the external buffer. Under these conditions the inside directed Fe^{2+} gradient is most likely to be the only driving force. An additional inward directed pH gradient stimulated this transport, whereas inversion of this gradient resulted in a near complete inhibition of transport. The Fe^{2+} influx observed with SMPs is primarily driven by the Fe^{2+} gradient and stimulated by the proton gradient and/or the membrane potential resulting from the pH gradient. Based on the stimulating role of the pH gradient we propose that Mrs3/4 proteins might exchange Fe^{2+} for OH^-, or – more likely – co-transport Fe^{2+} with H^+, similar to the mechanism described for the mammalian divalent metal ion transporter DMT1 in the plasma-membrane [30].

Several biological observations support the notion that the Fe^{2+} transport activity seen here with SMPs reflects a physiologically relevant function of *MRS3/4*: cells lacking Mrs3/4p (*mrs3/4Δ* mutant) fail to grow on iron-deplete media [2,16]. Furthermore, steady-state levels of *MRS4* mRNA are higher in cells grown on iron-free media than in those grown in iron-replete media (Wiesenberger, unpublished results). Consistent with this we observed increased Fe^{2+} uptake rates in SMPs originating from cells grown under iron starvation conditions. Finally, bio-available iron in mitochondria was reduced in *mrs3/4Δ* cells when grown under iron-depletion [3] and defects in mitochondrial iron acquisition can be compensated for by increasing or decreasing available iron in the cytoplasm [16].

We propose that the Mrs3/4p constitutes high affinity Fe^{2+} transporters which become essential only when other (yet unidentified) uptake systems of lower iron affinity cannot fulfil the demand for mitochondrial iron, e.g. in iron-depleted growth media.

Because *mrs3/4Δ* mutant SMPs failed to exhibit any significant Fe^{2+} uptake, we assume that these other iron uptake systems are not contributing to Fe^{2+} uptake into isolated inner mitochondrial membrane vesicles under the conditions applied here. They may be defective due to preparation of SMPs, may not be activated by the substrates provided in our assays (external Fe^{2+}, H^+, OH^-) or may require additional substrates, cofactors or a high membrane potential [31].

Surprisingly, we detected an Mrs3/4p-dependent transport of Cu^{2+} across SMP membranes. Transport rates for Cu^{2+} were comparable to those observed for Fe^{2+} but final concentrations inside SMPs remained significantly lower. It remains to be shown that this activity is of physiological relevance for mitochondria *in vivo*. Genetic studies on Cu^{2+} uptake into mitochondria had so far not uncovered any participation of Mrs3/4p. Mutant *mrs3/4Δ* cells have increased sensitivity to copper, but this is likely to result from activation of the transcription factor Aft1p in these cells which widely changes metal ion homeostasis [3,16].

Previous studies by the Winge group revealed the presence of a non-proteinaceous pool of copper in the mitochondrial matrix [32]. This matrix pool contains most of the mitochondrial copper as a Cu(I) complexed with a yet unknown ligand [32,33]. This ligand is a small fluorescent anionic molecule or metabolite which in its free form is abundant in the cytoplasm. These findings led Cobine et al. to propose that a Cu(I)–ligand complex forms in the cytoplasm and is translocated to the mitochondrial matrix, possibly by a mitochondrial carrier [33]. Mrs3/4 transport activities observed here are at variance with this model. In our assays Cu(II) is shown to serve as a substrate for Mrs3/4. Furthermore, our assays lacked ligands described by Cobine et al. equivalent to the added concentrations of copper, even if a small fraction might have been co-purified with the SMPs. It seems more plausible Cu^{2+} is – like Fe^{2+} – transported as a free ion in our assays but this activity of Mrs3/4p may not reflect their natural role in living cells.

A variety of physiological effects have been observed upon deletion or over-expression of *MRS3/4* genes. When overexpressed *MRS3* or

MRS4 partially restores Mg^{2+} uptake into mitochondria and respiratory growth of cells lacking Mrs2p, a Mg^{2+} channel located in the inner mitochondrial membrane [34–37]. Moreover, the temperature-sensitive growth of *mrs3/4Δ* strains can be suppressed by supplementation of media with 100 mM Mg^{2+} (Wiesenberger, unpublished results).

Cd^{2+} sensitivity is strongly increased in *mrs3/4Δ* strains and also in strains overexpressing Mrs3/4p. *mrs3/4Δ* strains are more resistant to Co^{2+} (and Mn^{2+}) while over-expression of Mrs3/4p causes hypersensitivity to Co^{2+}. Growth of *mrs3/4Δ* cells is reduced at elevated concentrations of copper [2,16], but more dependent on the presence of zinc than that of wild-type cells (Wiesenberger, unpublished results).

Mechanisms by which *MRS3/4* disruption or over-expression cause this variety of changes in cellular ion tolerance remain obscure. Transport of cations other than Fe^{2+} is not compatible with most of these observations (e.g. increased copper or cadmium sensitivity in *mrs3/4Δ* strains) and Zn^{2+} has been shown here not to be a substrate for Mrs3/4. Thus, the effects may be secondary to the deregulation of mitochondrial or cellular Fe^{2+} homeostasis which is known to affect expression of many genes. In fact, *MRS3* gene expression is under Zn^{2+} regulation [38] and mitochondrial Zn^{2+} concentrations correlate with the expression level of *MRS3/4* genes in iron-starved cells [3] while the copper effect has been attributed to activation of the Aft1 transcription factor [16]. Generally, cells may balance changes in cellular Fe^{2+} homeostasis by increasing or decreasing concentrations of other ions. Finally, Fe^{2+} and other divalent cations may compete for common binding sites and substitution of Fe^{2+} by other ions may have significant effects on cell growth (e.g. Co^{2+}, [39]).

Taken together we show here for the first time that iron is transported across the inner mitochondrial membrane. We believe, that the technique employed here is the method of choice both to show the involvement of Mrs3p and Mrs4p in Fe^{2+} transport across the inner mitochondrial membrane and to fully characterise this transport in terms of kinetics and transport specificity. The major advantage of using SMPs for transport studies over studying transport into intact mitochondria is the fact that the precise composition of the milieu on both sides of the membranes (inside and outside) can be set independently at will.

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