

The Family of *SMF* Metal Ion Transporters in Yeast Cells*

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Metal ions are vital for all organisms, and metal ion transporters play a crucial role in maintaining their homeostasis. The yeast (*Saccharomyces cerevisiae*) Smf transporters and their homologs in other organisms have a central role in the accumulation of metal ions and their distribution in different tissues and cellular organelles. In this work we generated null mutations in each individual *SMF* gene in yeast as well as in all combinations of the genes. Each null mutation exhibited sensitivity to metal ion chelators at different concentrations. The combination of null mutants $\Delta SMF1 + \Delta SMF2$ and the triple null mutant $\Delta 3SMF$ failed to grow on medium buffered at pH 8 and 7.5, respectively. Addition of 5 μM copper or 25 μM manganese alleviated the growth arrest at the high pH or in the presence of the chelating agent. The transport of manganese was analyzed in the triple null mutant and in this mutant expressing each Smf protein. Although overexpression of Smf1p and Smf2p resulted in uptake that was higher than wild type cells, the expression of Smf3p gave no significant uptake above that of the triple mutant $\Delta 3SMF$. Western analysis with antibody against Smf3p indicated that this transporter does not reach the plasma membrane and may function at the Golgi or post-Golgi complexes. The iron uptake resulting from expression of Smf1p and Smf2p was analyzed in a mutant in which its iron transporters *FET3* and *FET4* were inactivated. Overexpression of Smf1p gave rise to a significant iron uptake that was sensitive to the sodium concentrations in the medium. We conclude that the Smf proteins play a major role in copper and manganese homeostasis and, under certain circumstances, Smf1p may function in iron transport into the cells.

Transition metals are essential for many metabolic processes, and their homeostasis is crucial for life processes. Metal ion transporters play a major role in maintaining the correct concentrations of the various metal ions in the different cellular compartments. Recent studies of yeast (*Saccharomyces cerevisiae*) mutants revealed key elements in metal ion homeostasis, including novel transport systems. Several of the proteins discovered in yeast are highly conserved, and defects in some of the yeast mutants could be complemented by their human homologs (1–4). Some yeast and human homologous proteins were found to be related to copper and iron transport (5–7), among which the most ubiquitous are the *SMF* family of genes

encoding metal ion transporters (8–10). *SMF1* was originally cloned as a high copy number suppressor of a temperature-sensitive *mif1-1* mutant (11). Later it was shown that the growth arrest at 37 °C could be relieved by supplementing the media with Mn^{2+} or overexpressing *SMF1* that transports Mn^{2+} from the medium and elevates its concentration in the cytoplasm (8, 12, 13). The temperature-sensitive *mif1-1* mutant may have resulted from reduced stability of the processing peptidase under limited manganese concentrations in the medium (8). Further studies indicated the *SMF1* is a general metal ion transporter and can transport not only Mn^{2+} , Zn^{2+} , and Cu^{2+} (8), but also Fe^{2+} , Cd^{2+} , Ni^{2+} , and Co^{2+} (9, 12, 14). Yeast cells contain additional two genes of this family, *SMF2* and *SMF3*, and indirect evidence indicates that they are also broad range metal ion transporters but exhibit different specificity from *SMF1* (10), suggesting a specific function for each of them.

Expression of Smf1p in *Xenopus* oocytes demonstrated that this protein mediates H^+ -dependent divalent metal ion transport (14). In addition, a large Na^+ leak through Smf1p was observed, and sodium competed with the activity of metal ion uptake. Because the Smf family of proteins transports a wide range of divalent metal ions and specific and highly regulated transport systems exist in yeast for several of those metals (5–7), the function of the Smf family of proteins is not apparent. Our approach, since the discovery of these family members as metal ion transporters (8), was to identify conditions and yeast mutants that will help to elucidate specific functions of the various genes.

Here we report on the properties of deletion mutants in each of the genes encoding *SMF1*, *SMF2*, and *SMF3*, as well as the combination of multiple deletions, including a triple mutant lacking the three genes. Our study revealed that the Smf proteins take part in the transport of Cu^{2+} and Mn^{2+} into yeast cells, and in their absence a growth arrest occurs due to a shortage in these metal ions.

MATERIALS AND METHODS

Strains, Media, and Reagents—The “wild-type” that was used is *S. cerevisiae* W303 (*MAT α trp1 ade2 his3 leu2 ura3*). The other strains used in this work are: $\Delta SMF1$ (*MAT α ade2 his3 leu2 trp1 SMF1::URA3*); $\Delta SMF2$ (*MAT α ade2 his3 trp1 leu2 SMF2::URA3*); $\Delta SMF3$ (*MAT α ade2 his3 trp1 leu2 SMF3::URA3*); $\Delta SMF1+2$ (*MAT α ade2 his3 leu2 ura3 SMF1::URA3/FOA SMF2::URA3*); $\Delta SMF1+3$ (*MAT α ade2 his3 leu2 ura3 SMF1::URA3/FOA SMF3::URA3*); $\Delta SMF2+3$ (*MAT α ade2 his3 leu2 ura3 SMF2::URA3/FOA SMF3::URA3*); $\Delta SMF1+2+3$ (*MAT α ade2 his3 leu2 ura3 SMF1::URA3/FOA SMF2::URA3/FOA SMF3::URA3*). The yeast strain in which *FET3* and *FET4* genes were inactivated ($\Delta 2FET$) was the DEY 1453 (15).

The cells were grown in a YPD medium containing 1% yeast extract, 2% bacto-peptone, and 2% dextrose. For metal ion limitation experiments, the cells were grown in a medium containing 0.25% yeast ex-

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tract, 0.5% bacto-peptone 2% dextrose, 50 mM MES,¹ and the pH was usually adjusted to pH 6 by NaOH (16, 17). Agar plates were prepared by the addition of 2% agar to the YPD buffer medium at the given pH. Yeast transformation was performed as described previously (18), and the transformed cells were grown on minimal plates containing a 0.67% yeast nitrogen base, 2% dextrose, 2% agar, and the appropriate nutritional requirements.

Gene Disruption—The gene knockout of the new strains was performed as follows: All or part of the target gene was replaced by the selectable marker *URA3*, leaving flanking DNA sequences of about 0.3 kb. When PCR was used for the construct, the DNA fragments were cloned into the TA plasmid or pGEM-T Easy (Promega). Transformed colonies that grew on the selective medium were selected, checked by PCR for homologous recombination, and analyzed for their phenotype. The genes containing approximately 0.3-kb flanking sequences were cloned by PCR into YEP24 or YPN2 or BFG plasmids (17). Sequential gene disruption was obtained by inactivation of the *URA3* gene and selection on minimal plates containing 1 mg/ml 5-fluorouracil. The colonies that grew under this condition were analyzed for lack of growth on minimal plates without uracil. These yeast strains were used for subsequent gene disruption with a *URA3*-selectable marker. The various null mutants were analyzed for the disruption of each gene by PCR using one primer from the *URA3* gene and one primer flanking the interrupted gene.

The interruption of *SMF1* was performed as described previously (8). *SMF2* was interrupted by the PCR-only (HANNAH) method (19). The 5'- and 3'-flanking regions of the gene, as well as the selectable marker, were amplified by PCR using oligonucleotides that are partially complementary to yield, in a second PCR, a DNA fragment composed of 5'-SMF2 *URA3* 3'-SMF2. Most of the reading frame of the gene was deleted leaving DNA fragments encoding 14 and 18 amino acids at the N and C termini of the transporter, respectively. *SMF3* was interrupted by introducing the *URA3* gene in the *StyI* site of *SMF3*. The same constructs were used for sequential interruption of more than one *SMF* gene.

Yeast Transformation—Yeast transformation was performed either by the method of Ito *et al.* (18) or by a bench-top method according to Elble (20). Yeast cells were grown overnight in 5 ml of YPD medium (pH 5.5) to stationary phase. The cells were centrifuged for 10 s in an Eppendorf microcentrifuge at 13,000 rpm. 10 μ l of salmon sperm (10 mg/ml) was added to the pellet as a DNA carrier. Then about 1 μ g of plasmid or DNA construct was added. Finally, the pellet was suspended in 0.5 ml of medium containing 10 mM Tris, pH 7.5, 1 mM EDTA, 40% polyethylene glycol 4000, and 0.1 M lithium acetate. The suspension was incubated overnight at room temperature and plated on the appropriate plates (17).

DNA Isolation from Yeast—Yeast cells were grown in 5 ml of selective or YPD medium to stationary phase. The cells were harvested by centrifugation for 2 min at 2500 rpm. The pellet was suspended in 100 μ l of STET solution containing 50 mM Tris (pH 8), 50 mM EDTA, 5% Triton X-100, and 8% sucrose. Glass beads (about 0.2 g) were added, and the suspension was vortexed for 20 min. Then, an additional 100 μ l of STET solution was added, and the mixture was boiled for 3 min, cooled for 1 min on ice, and centrifuged for 10 min at 18,000 $\times g$. 100 μ l was removed from the supernatant and 50 μ l of 7.5 M ammonium acetate was added. The mixture was incubated for 1 h at -20°C and centrifuged for 10 min at 18,000 $\times g$. 100 μ l of supernatant was removed to a fresh tube, 200 μ l of cold ethanol was added, and the mixture was centrifuged for 30 min at 18,000 $\times g$. The pellet, containing the DNA, was washed with 70% ethanol and dissolved in 20 μ l of 10 mM Tris and 1 mM EDTA (pH 8).

Antibody Preparation and Western Analysis—Polyclonal antibody against Smf3p was obtained by injecting rabbits with a chimeric protein containing the maltose-binding protein and the hydrophilic sequence of amino acids 382–469 of the Smf3p. The DNA fragment encoding these amino acids was amplified by PCR with introduced *EcoRI* and *HindIII* restriction sites. The amplified DNA fragment was cloned in-frame to the maltose binding protein in the plasmid PMAL-C (New England BioLabs). Following sequence verification, 500 ml of bacterial culture was grown to OD 0.5 at 600 nm, induced with isopropyl-1-thio- β -D-galactopyranoside for 3 h, and harvested by centrifugation at 4000 $\times g$. The cells were disrupted by French press, and the protein was purified

by using a column containing maltose agarose. The fractions containing the chimeric protein were dissociated by SDS, loaded on preparative gel, and electrophoresed. The gel was briefly stained by Coomassie Blue, the identified protein band was cut out, and the fusion protein was electroeluted. About 0.25 mg of fusion protein was injected into rabbits as described previously (21). Antibody to Pma1p was raised in rabbits using the purified protein that was electroeluted from polyacrylamide gels as described previously (21–23). Antibody against Sed5p was a generous gift from Dr. Randy Schekman (University of California, Berkeley, CA).

The antibody detection system (ECL) was from Amersham Pharmacia Biotech. Western blots were performed according to the protocol of the ECL antibody detection system from the manufacturer. Samples were denatured by SDS sample buffer and electrophoresed on 12% polyacrylamide Mini gels (Bio-Rad) as described previously (24). Following electrotransfer at 0.5 A for 15 min, the nitrocellulose filters were blocked for 1 h in a solution containing 100 mM NaCl, 100 mM sodium phosphate (pH 7.5), 0.1% Tween 20, and 5% nonfat dried milk. Antibodies were incubated for 30 min at room temperature at a dilution of 1:1000 in a similar solution containing 2% dried milk. Following five washes in the same solution, peroxidase-conjugated second antibody or protein A was added to the filters. After incubation for 30 min and five washes with the same solution, the nitrocellulose filters were subjected to the ECL amplification procedure. The filters were exposed to Kodak X-Omat AR film for 5–60 s.

Membrane Preparations—Yeast cells were grown in 500 ml of YPD medium (pH 5.5) to OD 1 at 600 nm. The suspension was centrifuged at 3000 $\times g$ for 5 min, and the pellet was washed with 200 ml of water and then washed again with 1 M sorbitol. The cell wall was digested by 2.5 units of zymolyase in 10 ml of solution containing 10 mM HEPES, pH 7.5, and 1 M sorbitol. After 30-min incubation at 30 $^{\circ}\text{C}$, the suspension was centrifuged in 15-ml Corex tubes at 3000 $\times g$ for 5 min. 1 ml of glass beads was added to the pellet as well as 1 ml of solution containing 30 mM MOPS (pH 7), 1:100 protease inhibitor mixture (Sigma), 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and 1 mM EGTA. The suspension was vortexed five times for 30 s with incubation on ice for 30 s between each vortexing step. The solution was removed from the glass beads and placed in a new Corex tube. An additional 2 ml of the above solution was added to the tube with the glass beads, and the tube was vortexed briefly. The suspension was added to the previous one and centrifuged at 1000 $\times g$ for 5 min to give a pellet containing the cell debris and nuclei. The supernatant was centrifuged at 12,000 $\times g$ for 10 min, and the pellet was suspended in 0.3–0.5 ml of a solution containing 10 mM Hepes (pH 7.5) and 0.5 M sorbitol and stored as the mitochondrial fraction. The supernatant was centrifuged at 115,000 $\times g$ for 30 min, and the pellet was suspended in 0.3–0.5 ml of a solution containing 10 mM Tris-Cl (pH 7.5), 1 mM EDTA, 2 mM dithiothreitol, and 25% glycerol and stored as the membrane fraction at -80°C . Sucrose gradients were also used to estimate the relative density of various membrane fractions. The gradients were made as described in Lupashin *et al.* (25), except that gradients of 20–60% sucrose were used and the centrifugation was done for 14 h.

RESULTS

The SMF Family of Yeast Metal Ion Transporters—SMF1 encodes a hydrophobic protein of 63,258 Da with potentially eight to ten transmembrane domains. A search in the yeast genome data base with the Smf1p sequence revealed two homologous genes that were named *SMF2* and *SMF3*. These genes encode proteins of 59,758 Da and 51,778 Da, respectively, and contain a similar number of transmembrane domains. Fig. 1 shows the multiple alignment of the predicted amino acid sequences of the three members of the yeast *SMF* gene family. The three proteins exhibit about 50% identity to each other, and the main difference between them was found at their N terminus. At this end, Smf3p is shorter than Smf1p by 70 amino acids and by 51 amino acids from Smf2p. These extra pieces are highly populated by charged amino acids. Up to 12 transmembrane segments have been proposed to constitute DCT1, the mammalian homolog of Smf1p (26). We assume that the number of transmembrane segments will be similar in all the family members and 10 or 12 transmembrane segments are likely to exist in these transporters. Multiple alignment of amino acid sequences of family members from bacteria, yeast,

¹ The abbreviations used are: MES, 4-morpholineethanesulfonic acid; kb, kilobase(s); PCR, polymerase chain reaction; MOPS, 4-morpholinepropanesulfonic acid; TM, transmembrane domain; t-SNARE, target SNAP receptor.

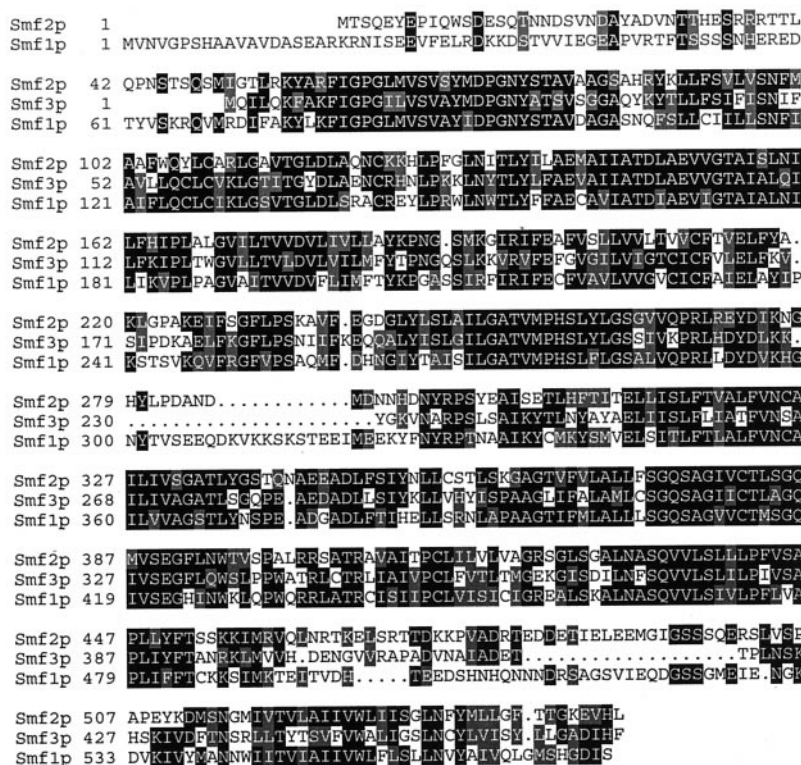


FIG. 1. Multiple alignment of the amino acid sequences of the SMF family members. The amino acid sequences of Smf1p through Smf3p were obtained from GenBank™ as the following reading frames: Smf1p, YOL122c; Smf2p, YHR050w; and Smf3p, YLR034c. The multiple alignment was done using the program Pileup. The Boxshade program was used for visualizing the results (gcg software package).

plants, insects, and mammals suggests that several conserved charged amino acids are present inside the membrane. Among them (using Smf1p amino acid sequence) are Asp-92 in TM1; Glu-160, Asp-167, and Glu-170 in TM3; and His-278 in TM6. The three negatively charged amino acids in TM3 face the same side of an α helix, suggesting a role in translocation of positively charged ions across the membrane. Obviously, the above structural and functional assumptions have to be examined by multiple experimental approaches.

Properties of Yeast Null Mutants in the Various SMF Genes—The main feature of the *SMF1* null mutant was its sensitivity to EGTA concentrations, toward which the parental wild-type strain was insensitive (8). The growth inhibition of Δ *SMF1* in the presence of EGTA could be alleviated by low concentrations of manganese or copper but not by any other metal ion. Deletion of the genes encoding *SMF1* and *SMF2* resulted in a double null mutant that was not able to grow at pH 8 but exhibited normal growth in YPD medium buffered at lower pH (27). We generated null mutations in each individual *SMF* gene as well as combinations of null mutations in these genes. The triple null mutant in the three *SMF* genes (Δ 3*SMF*) was highly sensitive to EGTA and was not able to grow on YPD medium buffered at pH 7.5. Table I summarizes the sensitivity and resistance of the various null mutants to the chelator EGTA, pH, various metal ions, high osmolarity, and oxidative stress (obtained by the addition of H_2O_2 to the medium). All the null *SMF* mutants exhibited some sensitivity to EGTA. Although Δ *SMF1* and Δ *SMF2* were quite sensitive to EGTA, Δ *SMF3* showed only marginal sensitivity to the chelating agent. The mutant strains Δ *SMF1*, Δ *SMF3*, and Δ *SMF1+3* exhibited resistance to relatively high Co^{2+} and Mn^{2+} concentrations in the medium. Δ *SMF2* showed resistance to osmotic stress induced by 0.9 M NaCl or 1.6 M glycerol or 1.5 or 1.7 M sorbitol, as well as relative resistance to Mn^{2+} , but only at pH 7.5. The resistance for certain metal ions can be explained in terms of reduced transport activity of these ions in the absence of one or more of the Smf metal ion transporters. It is more difficult to account for the sensitivity to metal ions in the

TABLE I
Phenotypes resulted from inactivation of each *SMF* gene and all the combination of *SMF* null mutations

The assay for resistance and sensitivity for metal ions was performed on a solid YPD medium buffered at pH 6 or the indicated pH. The medium was supplemented with the indicated metal ions in their chloride form at the following concentrations: Co^{2+} , 0.25, 0.5, 0.75 mM; Mn^{2+} , 1.5, 2, 2.5 mM; Zn^{2+} , 5, 6, 7 mM; Ni^{2+} , 1.5, 2, 2.5 mM. Sensitivity to EGTA was assayed as described in Fig. 2. Resistance to NaCl (0.9 M) and sorbitol (1.5 or 1.7 M) was analyzed at pH 6. Sensitivity or resistance to H_2O_2 was measured in liquid medium buffered at pH 6 at concentrations of 0.5 and 2 mM. Cells were inoculated to give a cell density of 0.2 OD at 600 nm, and the growth rate was measured every 2 h by following the increase in optical absorption at the same wavelength.

Mutant	Sensitivity	Resistance
Δ <i>SMF1</i>	EGTA	Co^{2+} , Mn^{2+}
Δ <i>SMF2</i>	EGTA, Zn^{2+} , Ni^{2+}	NaCl, sorbitol, H_2O_2
Δ <i>SMF3</i>	EGTA	Co^{2+} , Mn^{2+}
Δ <i>SMF1+2</i>	EGTA, Ni^{2+} , Co^{2+} , Mn^{2+}	
Δ <i>SMF1+3</i>	EGTA, Zn^{2+}	Co^{2+} , Mn^{2+}
Δ <i>SMF2+3</i>	EGTA	
Δ <i>SMF1+2+3</i> (Δ 3 <i>SMF</i>)	EGTA, H_2O_2 , pH 7.5, Zn^{2+}	

various null mutants. Thus Δ *SMF2* mutant was sensitive to Zn^{2+} and Ni^{2+} ; Δ *SMF1+2* was sensitive to Ni^{2+} , Co^{2+} , and Mn^{2+} ; and Δ *SMF1+3* was sensitive to Zn^{2+} . Apparently, disturbances in metal ion homeostasis may elicit pleiotropic effects through alteration and different distribution of the other metal ion transporters and/or signal transduction mediators (7, 9, 13, 28–31).

One of the key questions to be answered is how do seemingly unrelated metal ions elicit similar effect in mutants lacking one or more of the *SMF* genes? Inactivation of the *Drosophila* homolog of the *SMF1* gene resulted in a loss of taste behavior (32). Addition of manganese or iron to the food of these mutants corrected the defect in their taste behavior (33). Similarly, addition of micromolar concentrations of Cu^{2+} or Mn^{2+} relieved the growth arrest of the yeast mutant in which the

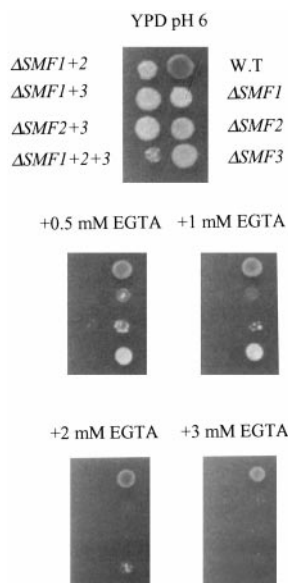


FIG. 2. Effect of EGTA on growth of the various combinations of *SMF* disruptant mutants. The buffered YPD plates (pH 6) were prepared as described under "Materials and Methods." The indicated concentrations of filter-sterile sodium-EGTA were added right before the pouring of the warm medium. Cultures of the various yeast strains were washed by sterile distilled water and 5 ml of the cell suspension, adjusted to 0.001 OD, were placed in the indicated positions. The plates were incubated for 2 days at 30 °C.

SMF1 gene was interrupted (8). To gain further information on the phenotype of the various *SMF* null mutants, we tested their growth under different EGTA concentrations. Fig. 2 shows a sensitivity order toward EGTA of $\Delta SMF1 > \Delta SMF2 > \Delta SMF3$ for the respective null mutants. The parental wild-type strain grew quite well at EGTA concentrations of up to 3 mM. Reduced growth was already detected at 0.5 mM EGTA for the mutants $\Delta SMF1$ and $\Delta SMF2$, and a complete growth arrest of these mutants was obtained at 2 mM EGTA. The mutant $\Delta SMF3$ grew well on plates containing 1 mM EGTA and a complete growth arrest of this mutant was obtained only at 3 mM EGTA. The triple mutant $\Delta SMF1+2+3$ was highly sensitive to the chelating agent and also exhibited pH sensitivity. It grew normally at pH 5.5, grew poorly at pH 6.5, and exhibited a complete growth arrest at pH 7.5 (Fig. 4).²

The growth arrest of the various *SMF* null mutants was alleviated by the inclusion of micromolar concentrations of Cu^{2+} or Mn^{2+} . Fig. 3 shows that at 3 mM EGTA, where all the *SMF* mutants fail to grow, only 5 μM Cu^{2+} were sufficient to promote growth. Similarly, 5 μM Mn^{2+} promoted growth of all the *SMF* mutants except for the triple mutant $\Delta SMF1+2+3$, which required about 10 μM Mn^{2+} for normal growth in the presence of 3 mM EGTA. The ability of this mutant to grow in the presence of EGTA was specific for Cu^{2+} and Mn^{2+} , and none of the other cations such as Zn^{2+} , Co^{2+} , Ni^{2+} , Fe^{3+} , Mg^{2+} , and Ca^{2+} could induce growth under these conditions. We were not able to analyze the effect of Fe^{2+} , because addition of ascorbic acid by itself promoted growth in the presence of EGTA. The effect of ascorbate may result from the reduction of the EGTA-bound Cu^{2+} into Cu^+ , which became available to an alternative copper uptake system.

The transport properties of the different Smf proteins were analyzed by expressing the various *SMF* genes in the triple null mutant $\Delta SMF1+2+3$ and assaying the transport of Mn^{2+} and Fe^{2+} by radioactive tracing. As shown in Fig. 5, expression of Smf1p or Smf2p in the triple null mutant restored the Mn^{2+}

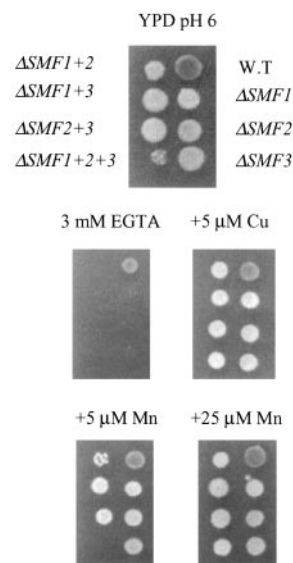


FIG. 3. Copper and manganese promote growth in the presence of EGTA of the various *SMF* disruptant mutants. The experimental procedure was as described in Fig. 2, except that the indicated $CuCl_2$ or $MnCl_2$ was added together with the sodium-EGTA.

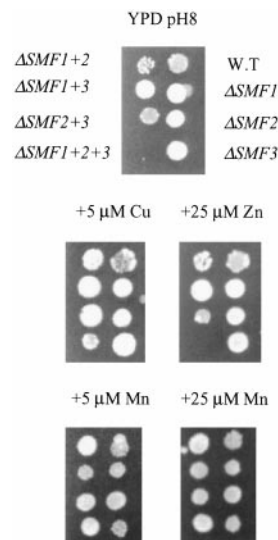


FIG. 4. The triple *SMF* disruptant mutant ($\Delta SMF1+2+3$) fails to grow at high pH and both copper and manganese promote growth under this condition. The experimental procedure was as described in Fig. 2, except that the medium was adjusted to pH 8, EGTA was omitted, and the indicated concentrations of $CuCl_2$, $MnCl_2$, or $ZnCl_2$ were added.

uptake activity above the wild-type level. Smf3 exhibited no transport activity and the short Smf1p (Smf1p-s), in which 68 amino acids were removed from its N terminus, exhibited 2- to 3-fold higher transport activity than either Smf1p or Smf2p. The lack of transport activity in the cells expressing Smf3p may be due to its different cellular distribution. Fig. 6 shows that the expressed Smf3p failed to reach the plasma membrane. Smf3p is present in the sucrose gradient fractions enriched with post-Golgi vesicles where Sed5p is present. Sed5p is a syntaxin (t-SNARE) homolog required to Golgi transport and is located in the Golgi membrane, which receives transport vesicles (34). Moreover, switching to nonpermissive temperature in the *sec6* mutant resulted in similar shifts in the gradient position of both proteins. The mutant cells were grown overnight at 25 °C, and then the temperature was raised to 37 °C for 2 h. The cells were harvested and disrupted, and their membranes

² A. Cohen, H. Nelson, and N. Nelson, unpublished results.

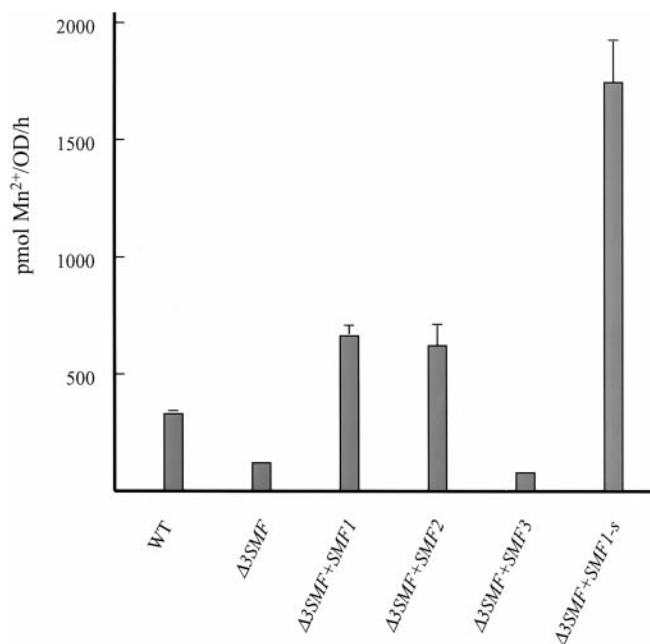


FIG. 5. Manganese uptake by the triple SMF disruptant mutant ($\Delta 3SMF$) expressing each of the SMF genes. The disruptant mutant ($\Delta 3SMF$) in each of the SMF genes was generated as described under "Materials and Methods." Mutant cells were transformed by shuttle vectors containing each of the SMF genes: *SMF1* in YEP24, *SMF2* in YEP24, *SMF3* in BFG, and *SMF1-s* in BFG. The transformed cells were grown on a minimal medium containing glucose and the appropriate nucleotides and amino acids and harvested at OD 1 at 600 nm. The cells were suspended in a medium containing 2% glucose, 25 mM Mes-Tris (pH 4), 10 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 100 mM choline chloride, and 1 μ M MnCl₂ to give a cell density of 0.2 OD at 600 nm. 0.5 ml of the cell suspension was placed on ice in an Eppendorf tube. The cells were precipitated by a short spin (13,000 rpm for 20 s), and 0.5 ml of the above medium (ice-cold) containing about 10,000 cpm of ⁵⁴Mn²⁺ was added to each tube. The cells were suspended by vortexing and incubated for 15 min at 30 °C. Following the incubation, the tubes were placed on ice to stop the reaction, and the cells were washed four times with 0.5 ml of ice-cold medium containing 1 mM MnCl₂. Finally, the cells were sedimented in an Eppendorf microcentrifuge, suspended in 50 μ l of water, and transferred to scintillation vials. 50 μ l of 1% SDS and 4 ml of scintillation fluid were added to the vials, and the radioactivity was counted in a Beckman scintillation counter. The uptake at 0 °C was deduced from each experiment.

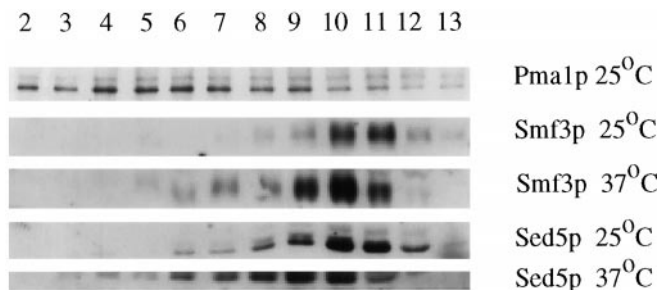


FIG. 6. Identification of Smf3p in the Golgi or post-Golgi membranes. The antibody against Smf3p was raised as described under "Materials and Methods." Wild type cells and the temperature-sensitive *sec6* mutant were grown at 25 °C and 37 °C in YPD media with 2% and 0.2% glucose, respectively (44). The cells were harvested and disrupted, and their membranes were collected by differential centrifugation as described under "Materials and Methods." They were then separated on sucrose gradients of 40–60%. Fourteen fractions were collected from the bottom of the tubes, and 5 μ l of the fraction was electrophoresed on SDS gels and subjected to Western analysis with the indicated antibodies.

were collected by differential centrifugation and separated on sucrose gradients of 40–60%. Western analysis revealed a similar shift in the position in the gradient of Sed5p and Smf3p.

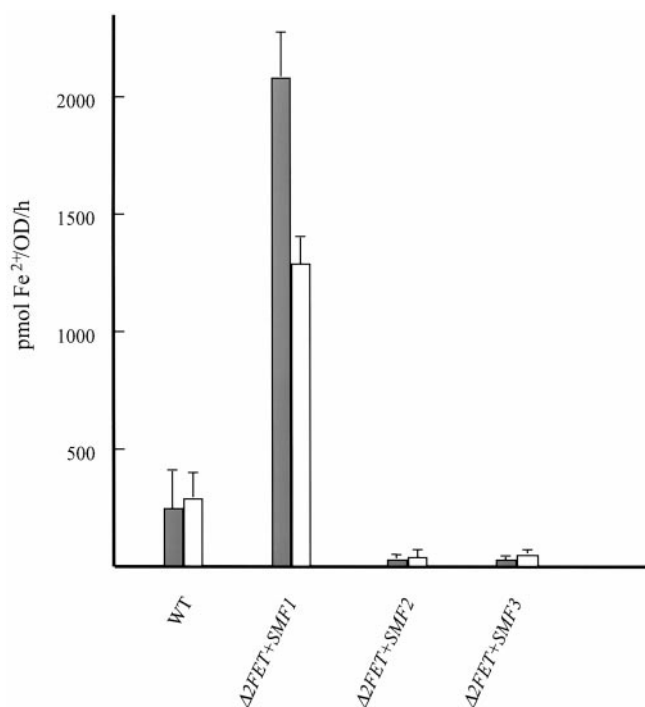


FIG. 7. Sodium inhibits FeCl₂ transport by Smf1p expressed in FET double disruptant mutant. The FET double mutant ($\Delta 2FET$) was transformed by shuttle vector containing each of the SMF genes as described in Fig. 5. The uptake experiment was similar to that of Fig. 5, except that 1 mM FeCl₂ and ⁵⁵Fe²⁺ replaced the manganese and 2 mM sodium ascorbate was added to the uptake medium to maintain the iron in its reduced form. The pH of the uptake medium was 6.5. The uptake medium contained either 100 mM choline chloride (gray columns) or 100 mM NaCl (white columns). The experiment was repeated twice, and the bars with standard deviation represent the mean of all the experiments.

This may suggest that Smf3p functions in the Golgi or post-Golgi compartment and is likely to be involved in maintaining Cu²⁺ and/or Mn²⁺ homeostasis in these organelles.

Previously we reported that Na⁺ inhibits metal ion transport by Smf1p and prevents growth of the wild type or $\Delta SMF2$ strains in the presence of EGTA (14). Addition of sodium to the manganese uptake system as described in Fig. 5 (even at pH 6.5) had little or no effect (not shown). Experiments with *Xenopus* oocytes revealed that the sodium inhibition of expressed Smf1p is highly dependent on the membrane potential and is drastically reduced at negative membrane potentials.³ Because yeast cells maintain high values of negative membrane potential, we turned to iron uptake for analyzing the effect of sodium on the metal ion uptake. Iron uptake into yeast cells is primarily mediated by a system involving Fet3p and Fet4p (30). However, it was demonstrated that alternative transport systems for iron may exist (35). To decrease the iron uptake by its specific transport systems, we utilized the double mutant ($\Delta 2FET$) in which *FET3* and *FET4* were inactivated. Fig. 7 depicts a direct effect of Na⁺ on the Fe²⁺ uptake activity of Smf1p. The expressed Smf2p and Smf3p in the double mutant $\Delta 2FET$ failed to show iron uptake above background. This observation suggests that Smf1p plays a role in an alternative iron uptake system.

The effect of different metal ions on manganese uptake by the Smf proteins expressed in the triple null mutant ($\Delta 3SMF$) was analyzed (Fig. 8). Expressed Smf3p exhibited no significant uptake above the background of the triple mutant. Expressed Smf1p and Smf2p showed similar properties. Recent experiments with Smf1p expressed in *Xenopus* oocytes demon-

³ A. Sacher, A. Cohen, and N. Nelson, manuscript in preparation.

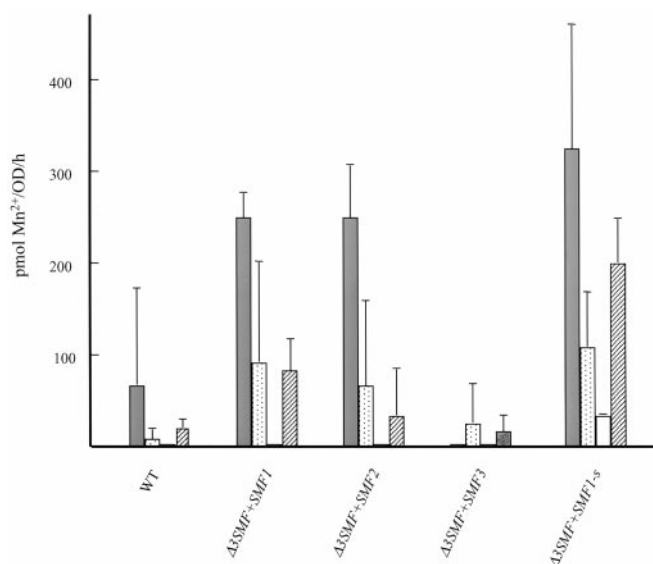


FIG. 8. **Inhibition of manganese transport activity of Smf proteins by different divalent cations.** The manganese uptake experiment was performed as described in Fig. 5, except that 0.1 mM metal chloride was added to the uptake medium. Gray columns, no addition; dotted columns, 0.1 mM CuCl₂; white columns, 0.1 mM ZnCl₂; dashed columns, 0.1 mM CoCl₂ was added. Each value is the mean of at least two independent assays, and the error bars indicate standard deviation.

strated Mn²⁺, Fe²⁺, and Co²⁺ transport with K_m values of 2, 5, and 11 μ M, respectively.³ The order of potency of inhibition by different divalent metal ions of ⁵⁴Mn²⁺ uptake was Cu²⁺ > Mn²⁺ > Fe²⁺ > Zn²⁺. As shown in Fig. 8, Zn²⁺ inhibited not only the manganese transport by Smf1p and Smf2p but also the transport by an additional unknown manganese transport system. The IC₅₀ of ⁵⁴Mn²⁺ uptake by Δ SMF expressing either Smf1p or Smf2p was about 2 μ M Cu²⁺ (not shown). Although copper and cobalt were likely to be transported by Smf1p and Smf2p, zinc was not transported and was likely to inhibit by blocking the transport pathway.

DISCUSSION

Redox reactions are fundamental life processes, and transition metals are essential for the function of most proteins involved in electron transport. The different metal ions may be grouped into redox active ions, such as Fe²⁺, Cu²⁺, Co²⁺, and to a less extent Mn²⁺, and non-redox-active ions such as Ca²⁺ and Zn²⁺. The redox-active ions normally function in enzymes that participate in redox reactions and the conversion of active oxygen-containing components. These processes require defined amounts of specific metal ions at the right position and in a timely fashion. Living cells compete for metal ions, but excess amounts are toxic and can cause damage to the very function that they serve, as well as to proteins and nucleic acids that are present in their proximity. Metal ion transporters provide an efficient tool for competition in the limited resources and the control over their accumulation storage and secretion. Genetic screens have identified several yeast genes that encode metal ion transporters or are involved in metal ion homeostasis transport (5–7, 10). One of those transporters, Smf1p, was shown to transport Mn²⁺ across the plasma membrane of yeast cells (8). SMF1 has originally been cloned as a high copy number suppressor of a temperature-sensitive *mif1-1* mutant (11). MIF1 (MAS1) and MAS2 (MIF2) encode the mitochondrial processing enhancing protein and the matrix processing peptidase, respectively (36–38). Later it was shown that three homologous genes, SMF1, SMF2, and SMF3, are present in the yeast genome (9, 10, 27). The presence of the three genes raises

some interesting questions about their expression and function.

In this work, we demonstrated that inactivation of each of the three SMF genes induced various degrees of EGTA sensitivity in the respective mutants. The growth arrest caused by EGTA in all the three mutants could be suppressed by the addition of 5 μ M Cu²⁺ or 25 μ M Mn²⁺ (Fig. 3). Addition of other divalent cations such as Zn²⁺, Co²⁺, Ni²⁺, Fe³⁺, Mg²⁺, and Ca²⁺ failed to induce growth under these conditions. The effect of Fe²⁺ could not be examined under the same conditions, because inclusion of ascorbic acid by itself suppressed the growth arrest caused by EGTA (not shown). However, the failure of high iron concentrations to induce growth and the lack of sensitivity to bathophenanthroline sulfonate suggest that the growth arrest was not due to iron limitation. It is well established that yeast growth requires a constant supply of copper (39, 40). However, very small amounts of copper are sufficient for sustaining growth, and it is argued that less than one free copper ion is present in each yeast cell (41). If so, how can disruption of each SMF gene cause copper shortage in the presence of EGTA? The answer is not apparent, especially in light of the presence of high affinity copper transport systems in the form of Ctr1p and Ctr3p (40). Inactivation of these proteins causes a severe disruption in iron homeostasis through the failure of Fet3p to assemble into the plasma membrane (30). Although the effect of Ctr1p and Ctr3p on iron transport was studied in detail, the function of these two proteins as copper transporters was not directly demonstrated. Inactivation of the three SMF genes resulted in a mutant strain (Δ SMF) that exhibited high sensitivity to several chelating agents such as EGTA, EDTA, bathophenanthroline sulfonate, and bathocuproinedisulfonic acid. In addition, the mutant failed to grow in YPD medium buffered at pH 7.5. Inclusion of only 5 μ M Cu²⁺ or 0.1 M ascorbate in the presence of the chelating agents or at pH 7.5 promoted the growth of the mutant cells. The effect of ascorbate can be explained by the reduction of Cu²⁺ to Cu⁺, thus making it more available to the cells. Therefore, our experiments suggest that the Smf proteins function in Cu²⁺ transport and that their inactivation causes copper shortage under oxidizing conditions.

Manganese also promoted growth of the various SMF null mutants in the presence of chelating agents or high pH. We demonstrated that both Smf1p and Smf2p function in Mn²⁺ uptake into yeast cells (8, 12). Most probably, Smf3p does not function in metal ion transport across the plasma membrane and may be involved in metal ion transport in the membranes of internal organelles such as the Golgi or post-Golgi vesicles and secondary endosomes (Fig. 6). These locations were shown to be critical for the assembly of iron-transporting proteins, especially through the incorporation of copper into iron oxidoreductases (30, 39). The Golgi and post-Golgi vesicles are also important for the correct glycosylation of membrane and secreted proteins. Recently it was demonstrated that the glycosylation complex is vital for yeast cells and that the activity of this complex is manganese-dependent (42, 43). Therefore, manganese deficiency in these compartments may result in growth arrest. Alternatively, the growth promotion of SMF null mutants in the presence of chelating agents by the addition of micromolar concentrations of manganese may result from the release of bound copper at the level of the Golgi and post-Golgi vesicles by the added manganese.

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REFERENCES

- Zhou, B., and Gitschier, J. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 7481–7486
- Hung, I. H., Suzuki, M., Yamaguchi, Y., Yuan, D. S., Klausner, R. D., and

- Gitlin, J. D. (1997) *J. Biol. Chem.* **272**, 21461–21466
3. Csere, P., Lill, R., and Kispal, G. (1998) *FEBS Lett.* **441**, 266–270
4. Tabuchi, M., Yoshida, T., Takegawa, K., and Kishi, F. (1999) *Biochem. J.* **344**, 211–219
5. Eide, D. J. (1998) *Annu. Rev. Nutr.* **18**, 441–469
6. Andrews, N. C., and Levy, J. E. (1998) *Blood* **92**, 1845–1851
7. Radisky, D. C., and Kaplan, J. (1999) *J. Biol. Chem.* **274**, 4481–4484
8. Supek, F., Supekova, L., Nelson, H., and Nelson, N. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 5105–5110
9. Supek, F., Supekova, L., Nelson, H., and Nelson, N. (1997) *J. Exp. Biol.* **200**, 321–330
10. Nelson, N. (1999) *EMBO J.* **18**, 4361–4371
11. West, A. H., Clark, D. J., Martin, J., Neupert, W., Hartl, F.-U., and Horwich, A. L. (1992) *J. Biol. Chem.* **267**, 24625–24633
12. Liu, X. F., Supek, F., Nelson, N., and Culotta, V. C. (1997) *J. Biol. Chem.* **272**, 11763–11769
13. Liu, X. F., and Culotta, V. C. (1999) *J. Biol. Chem.* **274**, 4863–4868
14. Chen, X.-Z., Peng, J.-B., Cohen, A., Nelson, H., Nelson, N., and Hediger, M. A. (1999) *J. Biol. Chem.* **274**, 35089–35094
15. Eide, D., Broderius, M., Fett, J., and Guerinot, M. L. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 5624–5628
16. Nelson, H., and Nelson, N. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 3503–3507
17. Noumi, T., Beltrán, C., Nelson, H., and Nelson, N. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 1938–1942
18. Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) *J. Bacteriol.* **153**, 163–168
19. Supekova, L., Supek, F., and Nelson, N. (1995) *J. Biol. Chem.* **270**, 13726–13732
20. Elble, R. (1992) *BioTechniques* **13**, 18–20
21. Nelson, N. (1983) *Methods Enzymol.* **97**, 510–523
22. Koland, J. G., and Hammes, G. G. (1986) *J. Biol. Chem.* **261**, 5936–5942
23. Cohen, A., Perzov, N., Nelson, H., and Nelson, N. (1999) *J. Biol. Chem.* **274**, 26885–26893
24. Nelson, H., Mandiyan, S., and Nelson, N. (1994) *J. Biol. Chem.* **269**, 24150–24155
25. Lupashin, V. V., Pokrovskaya, I. D., McNew, J., and Waters, M. G. (1997) *Mol. Biol. Cell* **8**, 2659–2676
26. Gunshin, H., Mackenzie, B., Berger, U. V., Gunshin, Y., Romero, M. F., Boron, W. F., Nussberger, S., Gollan, J. L., and Hediger, M. A. (1997) *Nature* **388**, 482–488
27. Pinner, E., Gruenheid, S., Raymond, M., and Gros, P. (1997) *J. Biol. Chem.* **272**, 28933–28938
28. Ooi, C. E., Rabinovich, E., Dancis, A., Bonifacino, J. S., and Klausner, R. D. (1996) *EMBO J.* **15**, 3515–3523
29. Yuan, D. S., Dancis, A., and Klausner, R. D. (1997) *J. Biol. Chem.* **272**, 25787–25793
30. Askwith, C., and Kaplan, J. (1998) *Trends Biochem. Sci.* **23**, 135–138
31. Li, L., and Kaplan, J. (1998) *J. Biol. Chem.* **273**, 22181–22187
32. Rodrigues, V., Cheah, P. Y., Ray, K., and Chia, W. (1995) *EMBO J.* **14**, 3007–3020
33. Orgad, S., Nelson, H., Segal, D., and Nelson, N. (1998) *J. Exp. Biol.* **201**, 115–120
34. Banfield, D. K., Lewis, M. J., Rabouille, C., Warren, G., and Pelham, H. R. (1994) *J. Cell Biol.* **127**, 357–371
35. Askwith, C. C., de Silva, D., and Kaplan, J. (1996) *Mol. Microbiol.* **20**, 27–34
36. Pollock, R. A., Hartl, F.-U., Cheng, M. Y., Ostermann, J., Horwich, A., and Neupert, W. (1988) *EMBO J.* **7**, 3493–3500
37. Yang, M., Jensen, R. E., Yaffe, M. P., Oppliger, W., and Schatz, G. (1988) *EMBO J.* **7**, 3857–3862
38. Witte, C., Jensen, R. E., Yaffe, M. P., and Schatz, G. (1988) *EMBO J.* **7**, 1439–1447
39. Dancis, A., Yuan, D. S., Haile, D., Askwith, C., Elde, D., Moehle, C., Kaplan, J., and Klausner, R. D. (1994) *Cell* **76**, 393–402
40. Dancis, A., Haile, D., Yuan, D. S., and Klausner, R. D. (1994) *J. Biol. Chem.* **269**, 25660–25667
41. Rae, T. D., Schmidt, P. J., Pufahl, R. A., Culotta, V. C., and O'Halloran, T. V. (1999) *Science* **284**, 805–808
42. Knauer, R., and Lehle, L. (1999) *J. Biol. Chem.* **274**, 17249–17256
43. Knauer, R., and Lehle, L. (1999) *Biochim. Biophys. Acta* **1426**, 259–273
44. Nakamoto, R. K., Rao, R., and Slayman, C. W. (1991) *J. Biol. Chem.* **266**, 7940–7949