A yeast manganese transporter related to the macrophage protein involved in conferring resistance to mycobacteria

FRANTISEK SUPEK*, LUBICA SUPEKOVA*, HANNAH NELSON†, and NATHAN NELSON††

*Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110; and †Department of Biochemistry, Tel Aviv University, Tel Aviv 69978, Israel

Communicated by Gottfried Schatz, Biozentrum der Universität Basel, Basel, Switzerland, January 2, 1996 (received for review August 7, 1995)

ABSTRACT  A novel Saccharomyces cerevisiae mutant, unable to grow in the presence of 12.5 mM EGTA, was isolated by replica plating. The phenotype of the mutant is caused by a single amino acid change (Gly149 to Arg) in the essential yeast gene CDC1. The mutant could be suppressed by overexpression of the SMF1 gene, which was isolated as an extragenic high-copy suppressor. The SMF1 gene codes for a highly hydrophilic protein and its deletion renders the yeast cells sensitive to low manganese concentration. In accordance with this observation, the smf1 null mutant exhibits reduced Mn2+ uptake at micromolar concentrations. Using a specific antibody, we demonstrated that Smf1p is located in the yeast plasma membrane. These results suggest that Smf1p is involved in high-affinity Mn2+ uptake. This assumption was also tested by overexpressing the SMF1 gene in the temperature-sensitive mutant of the mitochondrial processing peptidase (MAS1). SMF1 overexpression as well as addition of 1 mM Mn2+ to the growth medium complemented this mutation. This also suggests that in vivo Mas1p is a manganese-dependent peptidase. The yeast Smf1p resembles a protein from Drosophila and mammalian macrophages. The latter was implicated in conferring resistance to mycobacteria. A connection between Mn2+ transport and resistance or sensitivity to mycobacteria is discussed.

Several metal ions function as prosthetic groups that permit electron transport by valence changes. They also function as activators for numerous enzymes and physiological processes. Therefore, their controlled uptake and secretion are essential for the vitality of most cells. Multiple mechanisms are involved in metal ion uptake into eukaryotic cells. For example, while iron uptake into mammalian cells involves receptor-mediated endocytosis, in Saccharomyces cerevisiae an uptake system containing a reducing step functions in iron uptake across the plasma membrane (1). Calcium homeostasis is also maintained by the coordination of several transport systems including Ca2+-ATPases (2–4), Na+/Ca2+ (5, 6) and H+/Ca2+ exchangers (7, 8) as well as several other carriers and channels. Very little is known about the transport systems of other metal ions such as Mn2+ and Zn2+ that are essential for the life cycle of eukaryotic cells. These metals play a crucial role not only in processes involving electron transport but also in key functions such as mitochondrial biogenesis and DNA replication and transcription (9, 10). Because a single ion like Mn2+ can be involved in several completely different processes, a defect in its transporter may exhibit unrelated phenotypes, some of which can be supplemented by higher concentrations of Mn2+ as a nutrient. S. cerevisiae cells are quite resilient to stress of low divalent cation concentrations (11, 12). Wild-type cells can readily grow in the presence of 12.5 mM EGTA in a medium buffered at pH 6.0. Mutations in necessary metalloproteins will render the yeast cells sensitive to low metal ion concentrations, which stems from the presence of EGTA in the medium. To explore the transport systems of metal ions, we selected mutants that cannot grow in the presence of 12.5 mM EGTA but contain intact V-ATPase (13). We isolated a mutant in CDC1 that could not grow in the presence of 12.5 mM EGTA but could be complemented by addition of Mn2+ to the growth medium or by overexpression of the SMF1 gene.

EXPERIMENTAL PROCEDURES

Strains, Media, and Genetic Techniques. S. cerevisiae strains W303-1b (MATa, leu2, his3, ade2, trp1, ura3), W303-1a (MATa, leu2, his3, ade2, trp1, ura3), E20 (MATa, leu2-3, leu2-112, his3-11, his3-15, trp1-1, ura3-52, mas1), and CRB1-5A (MATa, leu2, his3-401, trp1, ura3-52, HOL1-1, mas1-ts) were used. EGTA-sensitive mutants were generated by exposing yeast cells to ethyl methanesulfonate (EMS) as described (14) and replica plating on YPD plates (pH 6.0) containing 50 mM Mes and 12.5 mM EGTA. The mutants denoted as csp (chelator-sensitive phenotype) were back-crossed to the wild-type strain as described elsewhere (15). Yeast transformations were performed according to Ito et al. (16) and transformants were selected on solid SC media (0.67% yeast nitrogen base (2% glucose/2% agar) supplemented with the appropriate auxotrophic nutrients and 0.1% Casamino acids.

DNA Manipulations and Gene Cloning. DNA manipulations were carried out as described (17). A Carlson/Botstein library was used for transformation of the csp2 mutant. Plasmids were recovered from yeast colonies and cloned into Escherichia coli as described (18). DNA sequencing was performed by the dideoxynucleotide termination method (19). A set of nested deletions was generated by the Erase a base system (Promega) and was used for sequencing the first strand. The second strand was sequenced by using oligonucleotide primers designed according to the sequence of the first strand.

SMF1 Gene Disruption. A yeast strain bearing a disruption of the SMF1 gene was constructed by a one-step gene disruption method (20). The Bgl II fragment (1123 bp) of the SMF1 gene in pSPORT was replaced by a fragment carrying the URA3 marker. After cutting the resulting plasmid with DraI, the isolated fragment was used for transformation of yeast cells (W303-1b). The disruption of the SMF1 gene was verified by isolation of chromosomal DNA from transformants followed by amplification of a corresponding region by PCR and restriction analysis of the amplified product. Furthermore, the disruptant strain was crossed with the wild-type strain and segregation of the URA3 marker was verified.

Isolation of Yeast Organelles. Yeast cultures were grown at 30°C in 5 liters of rich medium (2% bactopeptone/1% yeast extract) supplemented with either 2% glucose (for isolation of vacuoles and plasma membranes) or 2% galactose (for isolation of mitochondria). Vacuoles were isolated as described by

Abbreviations: BAPTA, bis(2-aminophenoxy)ethane-N,N',N''-tetracetate; EMS, ethyl methanesulfonate.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. U15929 (CSP1) and U15970 (CSP2)].

†To whom reprint requests should be addressed.
Uchida et al. (21), except that last two EDTA washes were omitted. Crude plasma membrane was prepared essentially as described by Goffeau and Dufau (22). Briefly, the collected yeast cells (30 g) were resuspended in 50 ml of breaking buffer (50 mM Tris acetate, pH 7.5, 1 mM MgCl₂, 0.25 M sucrose, 0.5 mM phenylmethylsulfonyl fluoride) and lysed by passage through a French pressure cell (SLM Aminco, Urbana, IL) at 20,000 psi. After two centrifugations at 1000 x g and one centrifugation at 3000 x g, each for 5 min, the final supernatant was adjusted to pH 5.2 with acetic acid. The precipitated mitochondria were removed by 2.5 min of centrifugation at 6500 rpm and the pH of the supernatant was brought back to 7.5 with NaOH. The crude plasma membranes were sedimented by centrifugation of the neutralized supernatant at 26,000 x g for 20 min. The resulting crude plasma membrane preparation was further purified by sucrose gradient centrifugation (23). To isolate mitochondria, 30 g of yeast cells were processed as described by Yaffe (24), including purification by centrifugation through a Percoll gradient.

Anti-Smf1p Antibody. To generate antibody against Smf1p, a DNA fragment coding for 81 N-terminal amino acids of Smf1p was cloned in-frame with the C terminus of the maltose binding protein gene in the pMAL-cRI vector (New England Biolabs). The fusion protein was purified on a maltose-agarose column according to the manufacturer’s instructions. The purified fusion protein was concentrated by acetone precipitation, dissolved in a solution containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.1% SDS. Antibodies were raised in guinea pigs as described (25). To prepare affinity-purified antibody, ~10 mg of the purified fusion protein was cross-linked to 1 ml of Affi-Gel 10 (Bio-Rad) in 0.2 M Mops (pH 7.5). Solid ammonium sulfate was added to 5 ml of serum to give 50% saturation. The resulting pellet was dissolved in 5 ml of a PBS solution containing 100 mM sodium phosphate (pH 7.5) and 100 mM NaCl and was dialyzed overnight against the same solution. The dialyzed antibody was passed 10 times through the column to which the fusion protein was bound. The column was washed with 50 ml of the PBS solution followed by the second wash with 25 ml of PBS solution containing 0.5 M NaCl. The antibody was eluted with 0.1 M glycine HCl (pH 2.8) and 0.5-ml fractions were collected into tubes containing 0.1 ml of 1 M Tris-HCl (pH 8.0). The second purification step consisted of passing the affinity-purified antibody through a column of Affi-Gel with bound maltose-binding protein, which was purified and cross-linked to the resin in the same way as the fusion protein. The flowthrough solution from the last purification step, containing the non-adsorbed antibody, was dialyzed against PBS solution containing 35% glycerol and stored at −20°C.

Western Immunoblot Analysis. Western analysis was performed as described (13), using the ECL detection system (Amersham) as recommended by the manufacturer.

Metal Uptake Assays. Cells were grown in a synthetic minimal medium (26), from which the following components were omitted: CuSO₄, FeCl₃, ZnSO₄, and CaCl₂. After reaching an OD₆₀₀ of 0.5, 100 ml of the cell culture was sedimented, washed once with water, and resuspended in 10 ml of assay buffer (50 mM Mes/NaOH, pH 6.0/2% glucose). The cell suspension was vigorously shaken for 30 min at 30°C and then incubated for 20 min on ice. The reaction mixtures were constituted on ice by mixing 50 µl of assay buffer containing the specified concentration of metal ions, 50 µl of cell suspension, 0.5 mM MnCl₂, and 50 µl of cell suspension. After incubation at 30°C or 0°C, the reactions were stopped by addition of 1 ml of ice-cold 50 mM Mes/NaOH (pH 6.0) containing 0.5 mM MnCl₂. Cells were filtered through GF/F filters (Whatman) and washed five times with 5 ml of ice-cold solution containing 0.5 mM MnCl₂. Radioactivity was measured in a Beckman scintillation counter and the results were calculated according to the radioactivity of the original solution disposed onto the filters. The results were expressed as the amount of MnCl₂ in nmol taken up by 1 ml of cell suspension of OD₆₀₀ = 1.0 per min. The uptake at 0°C was subtracted from the corresponding uptake values at 30°C.

RESULTS

Selection of Mutants with Defect in Metal Homeostasis. S. cerevisiae was shown to accumulate multiple transition metals, which are present in trace amounts in the natural environment. It was demonstrated that high-affinity uptake of these metals is mediated by specific uptake systems (12, 27, 28). To identify possible candidates of these transporters, ~15,000 EMS-mutagenized colonies were replica-plated on YPD medium (pH 6.0) supplemented with 12.5 mM EGTA. Screening yielded 16 csp mutants (chelator sensitive phenotypes), which were unable to grow in medium containing EGTA but could grow in unsupplemented medium (YPD, pH 6.0).

Mutant strain csp2 was characterized in greater detail. The mutant differed from the wild-type strain by its sensitivity to EGTA (Fig. 1) but not to bithiopanthenolindisulfonic acid or BAPTA. To identify a growth-limiting metal, plates containing EGTA were supplemented with the following metals: CaCl₂, CoCl₂, CuCl₂, MgCl₂, MnCl₂, NiCl₂, and ZnCl₂ at concentrations of 0.01, 0.05, 0.25, or 1.25 mM. Only MnCl₂ at 0.05 mM or higher concentration rescued the growth of the mutant on plates containing EGTA. Of all the metals studied only ZnCl₂ at 1 mM concentration, showed specific growth inhibition of the csp2 mutant. This inhibition could be fully reversed by supplementing the zinc-containing medium with 0.2 mM MnCl₂. Supplementation with CaCl₂, CoCl₂, CuCl₂, MgCl₂, or NiCl₂ did not have any effect.

The csp2 mutant was crossed with the parental wild-type strain and the segregation of EGTA and Zn²⁺ sensitivities was examined by tetrad analysis. As both phenotypes cosegregated in the crosses and followed the pattern 2+:2−, both defects appear to be caused by a single chromosomal mutation. Neither EGTA nor Zn²⁺ sensitivity could be observed in the heterozygous diploid, indicating that the mutation is recessive.

Isolation of SMF1 Gene by Complementation and the Phenotype of Its Null Mutant. The csp2 mutant was transformed with a yeast genomic library of an average insert size of 12 kb, constructed in high-copy 2-µm vector (YEp24). About 50,000 Ura⁺ transformants were divided into 14 independent pools and an aliquot of each pool was subjected to a second round of selection on YPD plates containing EGTA. Two different plasmids were isolated from these colonies and their ability to complement the csp2 mutant was verified. One of them, containing a 3.6-kb DNA fragment, was later identified as carrying the previously cloned SMF1 gene. It is a multicopy suppressor of a temperature-sensitive mutant defective in the function of mitochondrial processing peptidase (29). It is noteworthy that, when cloned into low-copy-number plasmid (YPN2), SMF1 failed to complement the csp2 mutant.

Inactivation of the SMF1 gene was reported to have no detectable effect on cell viability (30). Because our experiments indicated possible involvement of the SMF1 gene in metal homeostasis, we examined the phenotype of smfl null mutant cells under the same conditions as described for the csp2 mutant. The strain bearing the null allele of the SMF1 gene could not grow on YPD plates containing 12.5 mM EGTA and was more sensitive than the wild-type strain to the other tested chelators (BAPTA or bithiopanthenolindisulfonic acid). Two metal cations, Mn²⁺ and Cu²⁺ at concentration of 10 µM concentration or higher, were found to relieve the growth inhibition caused by 12.5 mM EGTA. The deletion mutant was also tested for sensitivity to high concentrations of metals in the medium. The mutant and wild-type strains were streaked on YPD plates (pH 6.0) containing increasing concentrations of metal ion supplement as CaCl₂,
was increased sensitivity of the latter to NiCl₂. While growth of the wild-type strain on plates was completely inhibited by 4 mM CoCl₂, CuCl₂, MgCl₂, MnCl₂, NiCl₂, and ZnCl₂. The only SMF1 gene from csp2 mutant following PCR amplification. Sequence analysis confirmed that the SMF1 gene from the csp2 mutant did not contain any mutation.

**SMF1 Gene Mediates High-Affinity Mn²⁺ Uptake.** The sensitivity of Δsmf1 strain to EGTA and NiCl₂ may indicate that the SMF1 gene codes for a plasma membrane metal transporter. Our experiments suggested that Mn²⁺ is a probable substrate for Smf1p. Therefore, we measured manganese uptake by yeast strains in which the SMF1 gene was disrupted as well as in the wild type and in transformed cells containing single or multiple copies of the gene. Fig. 4 shows the effect of NiCl₂ concentrations on the manganese uptake by those three strains. A smf1 null mutant exhibited reduced uptake activity in comparison with the wild-type strain. Moreover, the strain containing SMF1 on a multicopy plasmid had ~5-fold higher manganese uptake activity than the wild-type strain. This uptake is dependent on temperature and at 30°C was linear for at least 10 min (not shown). These results suggest that Smf1p functions in a high-affinity manganese uptake by yeast cells. They also indicate the presence of a low-affinity system operating at manganese concentrations of ~5 μM and higher.

**A mas1-ts Mutant Can Be Rescued by Mn²⁺.** The SMF1 gene was previously cloned as a multicopy suppressor of a temperature-sensitive mutation in the MIF1 (MAS1) gene (30). The MAS1 and MAS2 (MIF2) genes encode proteins that in a heterodimeric form constitute the yeast mitochondrial signal peptidase (32–34). In vitro experiments demonstrated that purified peptidase activity is dependent on Mn²⁺, Co²⁺, or Zn²⁺ (29). However, the identity of the in vivo cofactor(s) remains unknown. Based on our finding that Smf1p transports manganese, we proposed that overexpression of Smf1p might lead to an increased intracellular manganese concentration, which would stabilize the mutant metallopeptidase at 37°C and thus enable growth at this temperature. To test this hypothesis, different yeast strains bearing a mas1-ts mutation were transformed with a Yep24 plasmid carrying the SMF1 gene. Two of the transformed strains, E20 and CRB1–5A, were selected for membrane), β subunit of F-ATPase (marker for the mitochondria), and subunit C of V-ATPase (marker for the vacuole). Aside from a small amount of plasma membrane ATPase present in purified vacuolar membranes, immunological analysis did not reveal any significant cross-contamination among the purified organelles. When the same organellar preparations were probed with the affinity-purified antibody against Smf1p, the protein was found to be present exclusively in the plasma membrane fraction. Neither mitochondria nor vacuolar membranes contained significant amounts of Smf1p (Fig. 3). These findings together with the sensitivity of a Δsmf1 strain to EGTA and NiCl₂ indicate that Smf1p may directly participate in metal uptake.

**Δsmf1::URA3**

![Fig. 1.](Image 1) csp2 and Δsmf1 strains cannot grow in the presence of EGTA. The indicated yeast strains were spread onto YPD plates (pH 6.0) lacking (Control) or containing (+ EGTA) 12.5 mM EGTA. The plates were incubated at 30°C for 2 days.

Fig. 2. Specificity of affinity-purified anti-Smf1p antibody. Whole cell lysates from wild-type (lanes 1) and Δsmf1 (lanes 2) strains were electrophoresed on SDS/12.5% polyacrylamide gel and either stained with Coomassie brilliant blue R-250 (4) or transferred onto nitrocellulose membrane and decorated with the affinity-purified anti-Smf1p antibody (B). Molecular masses of standards (lane S) were (from the top) 112, 84, 53.2, 34.9, 28.7, and 20.5 kDa.
with 1 mM MnCl₂. In contrast, 0.5 mM CoCl₂ or 1 mM ZnCl₂ prevented suppression of E20 phenotype by multiple copies of the SMF1 gene in the wild-type strain, E20, CRB1–5A, or could not (CRB1–5A) suppress the defect of the correspond-
ing strain. In the next step, the wild-type strain, E20, CRB1–5A, and the latter two strains transformed with a Yep24 plasmid bearing the SMF1 gene were streaked on YPD plates supplemented with 1 mM MnCl₂, 0.5 mM CoCl₂, or 1 mM ZnCl₂ and incubated at either 23°C or 37°C. The results of the experiment are shown in Fig. 5. The ts E20 strain, in which a mas1-ts mutation could be suppressed by overexpression of SMF1, could also grow at 37°C in the presence of 1 mM MnCl₂. On the other hand, the mas1-ts mutation of CRB1–5A could not be suppressed either by the overexpression of SMF1 or by supplementation of the medium with 1 mM MnCl₂. In contrast, 0.5 mM CoCl₂ or 1 mM ZnCl₂ failed to suppress the lack of growth at 37°C. Moreover, 1 mM ZnCl₂ prevented suppression of E20 phenotype by multiple copies of the SMF1 gene inhibiting growth at 37°C (not shown). These experiments strongly suggest that Mn²⁺ is the in vivo cofactor of the mitochondrial processing peptidase.

Smf1p Is Related to Nramp-1 That Is Involved in Macrophage Resistance to Mycobacteria. SMF1 encodes a highly hydrophobic protein containing 575 amino acids with a molecular weight of 63,271. The amino acid sequence of the yeast protein (see Fig. 6) is 30% identical with the corresponding sequence of the human and mouse Bcg gene product (Nramp; GenBank database accession nos. D38171, L32185, X75355, and L13732) (35–38). Hydropathy analysis revealed 10 potential transmembrane domains where the N and C termini are located in the cytoplasmic face of the membrane (Fig. 6). A similar organization was proposed for the mouse and the recently discovered Drosophila malvolio (MVL) gene involved in taste behavior (39). Their relatively high degree of identity and structure suggests a similar function for these membrane proteins. In addition, a search in GenBank (July 1995) revealed homologous proteins in Caenorhabditis elegans (accession no. U23525) and plants (accession nos. D15268, T04467, and T13234). Moreover, a highly homologous gene was recently identified in Mycobacterium leprae (~32% identity over 200 amino acids; accession no. U15184), suggesting that this family of transporters is widespread from bacteria to humans. We suggest that the members of this gene family function in divalent cation transport.

The csp2 Mutant Resulted from a Single Amino Acid Change in the CDC1 Gene. The second isolated plasmid that suppressed the csp2 phenotype carried a DNA insert of ~12 kb. To localize a complementing gene, the csp2 mutant was transformed with deleted variants of the DNA fragment (cloned into Yep24 plasmid), and the transformants were evaluated for their ability to grow on plates supplemented with EGTA. This procedure reduced the complementing region to a 5.8-kb Bgl II fragment, which was subsequently sequenced (Fig. 7). The complementing gene (formally denoted as Csp2) was later found to be identical with CDC1, which is a yeast gene involved in double-strand break-induced intrachromosomal recombination (40). In contrast to SMF1, CDC1 could complement the csp2 mutant when cloned into low-copy plasmid.

A strain carrying a null allele of the CDC1 gene was constructed by transforming diploid strain W303 with the above Bgl II fragment (Fig. 7), in which part of the CDC1 gene (amino acids 136–265) was replaced by the LEU2 marker. Leu⁺ transformants were sporulated and dissected, and the progeny were examined for the Leu⁺ phenotype. Only two spores from each tetrad gave rise to colonies and all were of the Leu⁺ phenotype. Microscopic inspection of the spores having a disrupted CDC1 gene revealed that most of them underwent two or three cell divisions. This indicates that Cdc1p is essential for cell viability and the possible mutation of the CDC1 gene in the csp2 mutant did not completely inactivate the gene. Therefore, the CDC1 gene in the csp2 mutant was amplified by PCR and subcloned into centromeric Ycp50 plasmid, and its sequence revealed one G to A mutation replacing Gly¹⁴⁹ with Arg. To confirm the identity of the mutation, mutant and wild-type alleles of the CDC1 gene were cloned into Ycp50 vector as Nde I/HindIII DNA fragments (Fig. 7) and used for transforming the disruptant strain (CDC1::ade1::LEU2). The transformed diploid strain was sporulated and the tetrads were dissected. Dissection yielded mostly three or four colonies. The colonies were picked and replicated on minimal medium lacking leucine or uracil and YPD medium (pH 6.0) supplemented with 12.5 mM EGTA or 4 mM ZnCl₂. Both the wild-type and the Gly¹⁴⁹ to Arg alleles of the CDC1 gene could support growth of

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**Fig. 3.** Localization of Smf1p to the plasma membrane. The following purified subcellular organelles and membranes were analyzed for the presence of Smf1p: lanes 1, mitochondria; 2, plasma membrane; 3, vacuolar membrane. Blots were probed with antibodies raised against Smf1p (A), Pma1p (B), β subunit of F-ATPase (C), and subunit C of V-ATPase-Vma5p (D).

**Fig. 4.** ⁵²Mn²⁺ uptake by yeast strains bearing different copy number of the SMF1 gene. Values represent average of three independent measurements with each measurement done in duplicate. Open circles, wild-type strain; solid circles, smf1 null mutant; solid squares, wild-type strain harboring the SMF1 gene on the multicopy plasmid Yep24 (p11.1).

**Fig. 5.** Suppression of mas1 mutation by the SMF1 gene or MnCl₂. Yeast strains were streaked on the indicated plates (clockwise from the top) as follows: wild-type W303–1b, CRB1–5A, CRB1–5A transformed with p11.1, E20, and E20 transformed with p11.1 (for p11.1 plasmid description see legend to Fig. 4). Plates were incubated at the indicated temperature for 3 days.
the haploid Δcdcl disruptant strain. In agreement with the previous observation, the Leu+ phenotype occurred only when present in combination with the Ura+ phenotype. As expected, Leu+ colonies bearing the Gly149 to Arg allele on the plasmid exhibited sensitivity to 4 mM ZnCl2 or 12.5 mM EGTA. The disruptant haploid strain harboring a plasmid containing the wild-type CDC1 gene could grow under those conditions. This experiment clearly demonstrated that the CDC1 gene codes for an essential yeast protein and the mutant allele of this gene determines the observed phenotype of the csp2 strain. The mutant allele of the CDC1 gene bearing the Gly149 to Arg change was denoted a cdc1-200. The data suggest that CDC1 is a manganese-dependent protein.

**DISCUSSION**

Several lines of evidence indicate that Smf1p mediates a high affinity Mn2+ uptake by yeast cells. First, smf1 null mutant cells are sensitive to EGTA but not to specific chelators of calcium and iron. The EGTA sensitivity was suppressed by supplementing the medium with low concentrations of MnCl2, suggesting the transporter's possible specificity. Second, overexpression of Smf1p suppressed EGTA sensitivity of the smf1 mutant, which is unable to grow with limited Mn2+ concentrations in the growth medium. This indicated that increased uptake of Mn2+ by the cells overexpressing Smf1p repaired the defect. Third, the smf1 null mutant cells exhibited a significant decrease in Mn2+ uptake. On the other hand, the presence of SMF1 at multiple copies stimulated Mn2+ uptake >5-fold.

The uptake of Mn2+ appeared to be mediated by two components. The first system had high affinity for Mn2+ and was apparently mediated by Smf1p. The second system primarily operated at Mn2+ concentrations higher than 10 μM and was unsaturable (data not shown). The plasma membrane location of Smf1p was in accord with its proposed function. We found that Mn2+ uptake was inhibited by Zn2+ (not shown), suggesting that Zn2+ competes with Mn2+ transport mediated by Smf1p. This observation could explain the growth arrest of csp2 mutant cells by a high concentration of Zn2+ and supports the conclusion that the csp2 mutant requires a higher intracellular concentration of Mn2+ than the wild type.

**FIG. 6.** Smf1p is related to Nramp from mammalian macrophages. Amino acid sequence of Smf1p is shown. Ten potential transmembrane helices are indicated by numbers 1–10. Identical amino acids in Smf1p and Nramp are indicated by boldface letters. Potential glycosylation sites are indicated by asterisks.

**FIG. 7.** Open reading frames in the Bgl II DNA fragment containing the CDC1 gene. Arrows indicate relative orientations of open reading frames (ORF). Restriction sites used in subcloning experiments are indicated.
is identical with the Ity and the Lsh gene conferring resistance to infection by Salmonella typhimurium and Leishmania donovani, respectively (41, 42). Although the cloning of Ntamp identified the gene responsible for resistance of mice to mycobacteria, its function is unknown. Because of a limited sequence identity between Ntamp and malvolio proteins with respiratory activity is thought to be partly mediated by microbicidal superoxide dismutase (SOD) (45), which contains Mn$^{2+}$ and/or Zn$^{2+}$ transporters. Fig. 8 depicts a proposed model for the role of Ntamp in macrophage defense against microbial invasion. Following the phagocytosis of a parasite into the phagosome, the macrophage produces reactive oxygen and/or nitrogen intermediates that are toxic for the internalized bacteria (43, 44). Phagosomes contain several plasma membrane proteins of which one may be the Nramp protein. The yeast homologue, transports Mn$^{2+}$ and, after formation of the phagosome, to deplete its lumen of Mn$^{2+}$ and, possibly function of Nramp and malvolio proteins as metal ion transporters. Fig. 8 depicts a proposed model for the role of Nramp in macrophage–pathogen interaction. Ntamp is proposed to function in Mn$^{2+}$ uptake by the macrophage and, after formation of the phagosome, to deplete its lumen of Mn$^{2+}$. Directionality of flow of Mn$^{2+}$ and O$_2^-$ is indicated by arrows. N, macrophage nucleus; A, superoxide dismutase; B, catalase.

![Diagram of macrophage and mycobacterium interaction](image)

**FIG. 8. A proposed role of Nramp in macrophage–pathogen interaction.** Nramp is proposed to function in Mn$^{2+}$ uptake by the macrophage and, after formation of the phagosome, to deplete its lumen of Mn$^{2+}$. Directionality of flow of Mn$^{2+}$ and O$_2^-$ is indicated by arrows. N, macrophage nucleus; A, superoxide dismutase; B, catalase.