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

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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 (Gly^{149} 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 hydrophobic protein and its deletion renders the yeast cells sensitive to low manganese concentration. In accordance with this observation, the *smf1* null mutant exhibits reduced Mn^{2+} 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 Mn^{2+} 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 Mn^{2+} 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 Mn^{2+} 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 Ca^{2+}-ATPases (2–4), Na^+/Ca^{2+} (5, 6) and H^+/Ca^{2+} 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 Mn^{2+} and Zn^{2+} 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 Mn^{2+} 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 Mn^{2+} 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 Mn^{2+} to the growth medium or by overexpression of the *SMF1* gene.

EXPERIMENTAL PROCEDURES

Strains, Media, and Genetic Techniques. *S. cerevisiae* strains W303-1b (*MATα, leu2, his3, ade2, trp1, ura3*), W303-1a (*MATa, leu2, his3, ade2, trp1, ura3*), E20 (*MATα, leu2-3, leu2-112, his3-11, his3-15, trp1-1, ura3-52, mas1*), and CRB1-5A (*MATa, leu2, his3, his4-401, 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 *Dra* I, 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

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Abbreviations: BAPTA, bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetate; 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*)].

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Uchida et al. (21), except that last two EDTA washes were omitted. Crude plasma membrane was prepared essentially as described by Goffeau and Dufuor (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 $MgCl_2$/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 × *g* and one centrifugation at 3000 × *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 × *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·NaOH (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: $CuSO_4$, $FeCl_3$, $ZnSO_4$, and $CaCl_2$. After reaching an OD_{600} 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 $MnCl_2$ containing $^{54}Mn^{2+}$ (39.5 mCi/mg, total manganese; 1 Ci = 37 GBq; DuPont/New England Nuclear) 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 $MnCl_2$. Cells were filtered through GF/F filters (Whatman) and washed five times with 5 ml of ice-cold solution containing 0.5 mM $MnCl_2$. 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 $MnCl_2$ in nmol taken up by 1 ml of cell suspension of OD_{600} = 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 phenotype), 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 bathophenanthrolinedisulfonic acid or BAPTA. To identify a growth-limiting metal, plates containing EGTA were supplemented with the following metals: $CaCl_2$, $CoCl_2$, $CuCl_2$, $MgCl_2$, $MnCl_2$, $NiCl_2$, and $ZnCl_2$ at concentrations of 0.01, 0.05, 0.25, or 1.25 mM. Only $MnCl_2$ at 0.05 mM or higher concentration rescued the growth of the mutant on plates containing EGTA. Of all the metals studied only $ZnCl_2$, 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 $MnCl_2$. Supplementation with $CaCl_2$, $CoCl_2$, $CuCl_2$, $MgCl_2$, or $NiCl_2$ did not have any effect.

The *csp2* mutant was crossed with the parental wild-type strain and the segregation of EGTA and Zn^{2+} 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 Zn^{2+} 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 *smf1* 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 (Fig. 1), but it was not more sensitive than the wild-type strain to the other tested chelators (BAPTA or bathophenanthrolinedisulfonic acid). Two metal cations, Mn^{2+} and Cu^{2+} 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 $CaCl_2$,

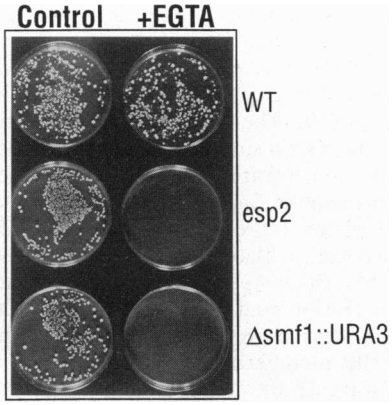

FIG. 1. *csp2* and Δ*smf1* strains cannot grow in the presence of EGTA. The indicated yeast strains were spread onto YPD plates (pH 6.0) lacking (Control) or containing (+EGTA) 12.5 mM EGTA. The plates were incubated at 30°C for 2 days.

$CoCl_2$, $CuCl_2$, $MgCl_2$, $MnCl_2$, $NiCl_2$, and $ZnCl_2$. The only observed difference between the wild-type and Δ*smf1* strains was increased sensitivity of the latter to $NiCl_2$. While growth of the wild-type strain on plates was completely inhibited by 4 mM $NiCl_2$, growth of the deletion mutant was abolished by a 2 mM concentration of the same metal. This phenomenon, together with the ability to complement the *csp2* mutant only when present in high-copy-number plasmid, suggested that the *SMF1* gene was distinct from the gene giving the Csp^- phenotype of the *csp2* strain. To answer this question, the *SMF1* gene was cloned from *csp2* mutant following PCR amplification. Sequence analysis confirmed that the *SMF1* gene from the *csp2* mutant did not contain any mutation.

Smf1p Is a Plasma Membrane Protein. West *et al.* (30) reported on experiments with epitope-tagged Smf1p, introduced into the yeast cells on a multicopy plasmid, that indicated the presence of the tagged protein in purified mitochondria. Because overexpression of protein may lead in some cases to its mislocalization and/or Smf1p could have been present in the mitochondrial preparation as a result of contamination by some other organelle, we decided to reexamine the subcellular localization of Smf1p. Fig. 2 shows a control experiment, where the whole cell lysates prepared from the wild-type and Δ*smf1* cells were separated on a polyacrylamide gel, transferred to a nitrocellulose membrane, and decorated with affinity-purified antibody raised against Smf1p. While Δ*smf1* cells did not contain protein crossreacting with the antibody, a single immunoreactive species with an apparent molecular mass of 65 kDa was observed in the wild-type cell lysate (Fig. 2). This shows the high specificity of the affinity-purified antibody for Smf1p. To identify the correct location of Smf1p, we applied the cell fractionation approach that is commonly used in yeast cell biology studies (31). Yeast spheroplasts were lysed in triethanolamine buffer and their membrane fractions were separated on sucrose gradients (60–35%). Membrane-specific markers were used for identification of the various organelles and membranes in the gradient fractions. NADPH-dependent cytochrome *c* reductase and GDPase were used to identify the endoplasmatic reticulum, ER and Golgi membranes, respectively. While the Smf1p fractionated in the middle of the gradient the ER membranes appeared near the bottom and the Golgi near the top of the gradient (not shown). This clear separation ruled out Smf1p as ER or Golgi component. To obtain better identification for the location of Smf1p, purified preparations of mitochondria, plasma membrane, and vacuolar membranes were subjected to Western analysis with antibodies against marker proteins and Smf1p. The markers used for identification of the various membrane preparations were yeast plasma membrane ATPase (marker for the plasma 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, immunoanalysis 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 Δ*smf1* strain to EGTA and $NiCl_2$ indicate that Smf1p may directly participate in metal uptake.

***SMF1* Gene Mediates High-Affinity Mn^{2+} Uptake.** The sensitivity of Δ*smf1* strain to EGTA and $NiCl_2$ may indicate that the *SMF1* gene codes for a plasma membrane metal transporter. Our experiments suggested that Mn^{2+} 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 $MnCl_2$ 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 Mn^{2+}. 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 Mn^{2+}, Co^{2+}, or Zn^{2+} (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

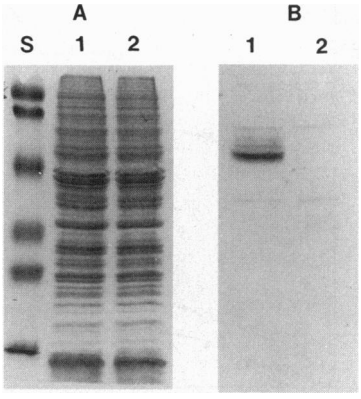

FIG. 2. Specificity of affinity-purified anti-Smf1p antibody. Whole cell lysates from wild-type (lanes 1) and Δ*smf1* (lanes 2) strains were electrophoresed on SDS/12.5% polyacrylamide gel and either stained with Coomassie brilliant blue R-250 (*A*) or transferred onto nitrocellulose membrane and decorated 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.

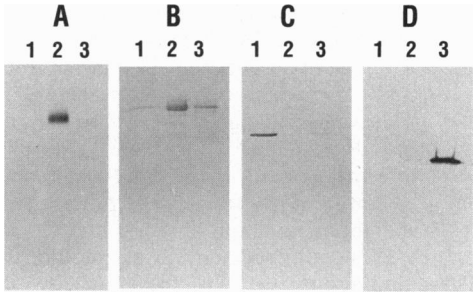

FIG. 3. Localization of Smf1p to the plasma membrane. The following purified subcellular organelles and membranes were analyzed for the presence of Smf1p: lanes 1, mitochondria; 2, plasma membrane; 3, vacuolar membrane. Blots were probed with antibodies raised against Smf1p (*A*), Pma1p (*B*), β subunit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

further analysis. Yep24 plasmid bearing *SMF1* either could (E20) or could not (CRB1–5A) suppress the *ts* defect of the corresponding 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 $MnCl_2$, 0.5 mM $CoCl_2$, or 1 mM $ZnCl_2$ 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 $MnCl_2$. 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 $MnCl_2$. In contrast, 0.5 mM $CoCl_2$ or 1 mM $ZnCl_2$ failed to suppress the lack of growth at 37°C. Moreover, 1 mM $ZnCl_2$ prevented suppression of E20 phenotype by multiple copies of the *SMF1* gene inhibiting growth at 37°C (not shown). These experiments strongly suggest that Mn^{2+} 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.

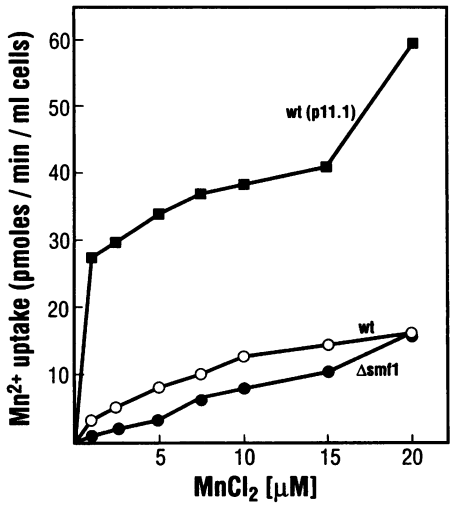

FIG. 4. $^{54}Mn^{2+}$ uptake by yeast strains bearing different copy number of the *SMF1* gene. Values represent average of three independent measurements with each measurement done in duplicate. Open circles, wild-type strain; solid circles, *smf1* null mutant; solid squares, wild-type strain harboring the *SMF1* gene on the multicopy plasmid Yep24 (p11.1).

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 Gly^{149} 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/*Hin*dIII DNA fragments (Fig. 7) and used for transforming the disruptant strain (*CDC1*/Δ*cdc1*::*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 $ZnCl_2$. Both the wild-type and the Gly^{149} to Arg alleles of the *CDC1* gene could support growth of

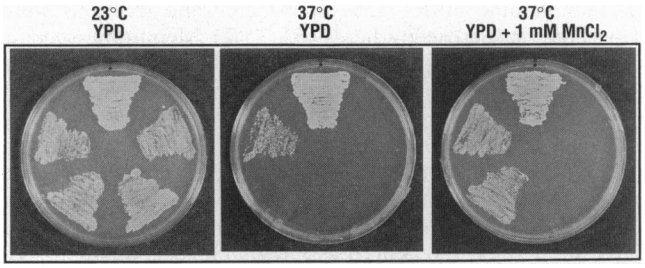

FIG. 5. Suppression of *mas1* mutation by the *SMF1* gene or $MnCl_2$. Yeast strains were streaked on the indicated plates (clockwise from the top) as follows: wild-type W303-1b, CRB1-5A, CRB1-5A transformed with p11.1, E20, and E20 transformed with p11.1 (for p11.1 plasmid description see legend to Fig. 4). Plates were incubated at the indicated temperature for 3 days.

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MVNVGPSHAA VAVDASEARK RNISEEVFEL RDKKDSTVVI EGEAPVRTFT SSSSNHERED TYVSKRQVMR DIFAKYLKFI GPGLMVSVAY IDPGNYSTAV  100
                                                                                       1
DAGASNQFSL LCIILLSNFI AIFLQCLCIK LGSVTGLDLS RACREYLPRW LNWTLYFFAE CAVIATDIAE VIGTAIALNI LIKVPLPAGV AITVVDVFLI  200
            2                                                                  3
MFTYKPGASS IRFIRIFECF VAVLVVGVCI CFAIELAYIP KSTSVKQVFR GFVPSAQMFD HNGIYTAISI LGATVMPHSL FLGSALVQPR LLDYDVKHGN  300
                                4                                                      5
YTVSEEQDKV KKSKSTEEIM EEKYFNYRPT NAAIKYCMKY SMVELSITLF TLALFVNCAI LVVAGSTLYN SPEADGADLF TIHELLSRNL APAAGTIFML  400
                                                                  6                                       7
ALLLSGQSAG VVCTMSGQIV SEGHINWKLQ PWQRRLATRC ISIIPCLVIS ICIGREALSK ALNASQVVLS IVLPFLVAPL IFFTCKKSIM KTEITVDHTE  500
                                                      8                              9
EDSHNHQNNN DRSAGSVIEQ DGSSGMEIEN GKDVKIVYMA NNWIITVIAI IVWLFLSLLN VYAIVQLGMS HGDIS   575
                                                      10
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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.

the haploid Δ*cdc1* 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 Gly^{149} to Arg allele on the plasmid exhibited sensitivity to 4 mM $ZnCl_2$ 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 Gly^{149} 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 Mn^{2+} 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 $MnCl_2$, suggesting the transporter's possible specificity. Second, overexpression of Smf1p suppressed EGTA sensitivity of the *csp2* mutant, which is unable to grow with limited Mn^{2+} concentrations in the growth medium. This indicated that increased uptake of Mn^{2+} by the cells overexpressing Smf1p repaired the defect. Third, the *smf1* null mutant cells exhibited a significant decrease in Mn^{2+} uptake. On the other hand, the presence of *SMF1* at multiple copies stimulated Mn^{2+} uptake >5-fold.

The uptake of Mn^{2+} appeared to be mediated by two components. The first system had high affinity for Mn^{2+} and was apparently mediated by Smf1p. The second system primarily operated at Mn^{2+} 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 Mn^{2+} uptake was inhibited by Zn^{2+} (not shown), suggesting that Zn^{2+} competes with Mn^{2+} transport mediated by Smf1p. This observation could explain the growth arrest of *csp2* mutant cells by a high concentration of Zn^{2+} and supports the conclusion that the *csp2* mutant requires a higher intracellular concentration of Mn^{2+} than the wild type.

SMF1 has also been cloned as a high copy number suppressor of a temperature-sensitive *mif1-1* mutant (30). *MIF1* (*MAS1*) and *MAS2* (*MIF2*) encode the processing enhancing protein and the matrix processing peptidase, respectively. The two proteins function as a heterodimer to form the active holoenzyme of mitochondrial processing peptidase (29). It has been reported that isolated mitochondria from a strain bearing epitope-tagged *SMF1* contained a cross-reactive material recognized by the anti-tag antibody (30). It was concluded that Smf1p is located in the mitochondrial membrane. Using Smf1p specific antibodies, we showed that the protein is located on the yeast plasma membrane. We suggest an alternative explanation for suppression of the *mif1-1* mutation by the function of Smf1p. The activity of the purified peptidase is inhibited by chelators such as EDTA or orthophenanthroline and is stimulated by Mn^{2+}, Zn^{2+}, or Co^{2+} (29). Since we demonstrated that Smf1p is a Mn^{2+} transporter, its overexpression could lead to increased cytoplasmic Mn^{2+} concentration. This elevated internal Mn^{2+} concentration exerted a greater stability for the mutated peptidase, which is known to require a divalent cation for its activity. In accordance with this hypothesis, we demonstrated that 1 mM $MnCl_2$ could suppress the *ts* phenotype of the *mas1* mutant. This suppression was allele specific because the same allele suppressed by *SMF1* could also be suppressed by 1 mM $MnCl_2$. Neither 1 mM Zn^{2+} nor 0.5 mM Co^{2+} could reverse the *ts* defect of the mutant, suggesting that Mn^{2+} functions as the *in vivo* cofactor of the metallopeptidase.

Similarly, Cdc1p may be a Mn^{2+}-dependent cell division cycle protein. The *csp2* mutant exhibits very similar characteristics to the *mas1* mutant with the exception of their growth inhibiting conditions (which are EGTA and 37°C, respectively). In both cases the block can be relieved by supplementing the medium with Mn^{2+}. We demonstrated that the Csp^- phenotype of the *csp2* mutant is caused by the a missense mutation in the *CDC1* gene. The Gly^{149} to Arg change apparently reduced the affinity of Cdc1p to Mn^{2+} and rendered the mutant sensitive to EGTA. Similarly, low internal Mn^{2+} concentration rendered the *mif1-1* mutant's protease less stable and therefore temperature sensitive.

We observed that *SMF1* shares homology with the mouse *Nramp* gene (35). *Nramp* (*Bcg*) was cloned as a gene responsible for mouse resistance to infection with mycobacteria and

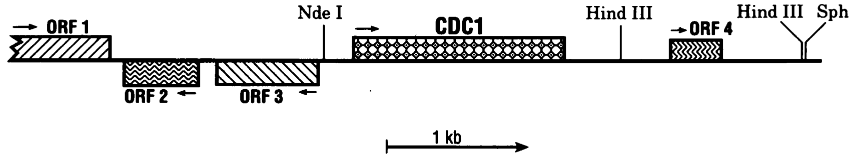

FIG. 7. Open reading frames in the *Bgl* II DNA fragment containing the *CDC1* gene. Arrows indicate relative orientations of open reading frames (ORF). Restriction sites used in subcloning experiments are indicated.

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 *Nramp* identified the gene responsible for resistance of mice to mycobacteria, its function is unknown. Because of a limited sequence identity between Nramp and malvolio proteins with nitrate transporter from *Aspergillus*, it was suggested that the *Drosophila* and mammalian proteins also function in NO_2^- or NO_3^- transport (35, 39). Although this suggestion is still feasible, the identification of Smf1p as the yeast Nramp homologue, and a possibly similar function of those two proteins, raises an alternative explanation for the involvement of Nramp in the resistance or sensitivity of macrophages to mycobacteria. We propose that the mammalian and insect membrane proteins, like the yeast transporter, are Mn^{2+} and/or Zn^{2+} transporters. Fig. 8 depicts a proposed model for the role of Nramp 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 survival of the pathogen during the burst of macrophage respiratory activity is thought to be partly mediated by microbial superoxide dismutase (SOD) (45), which contains Mn^{2+} or Fe^{2+} in its active center. We propose that Nramp, like its yeast homologue, transports Mn^{2+} from the extracellular milieu into the cytoplasm of a macrophage and, after the generation of the phagosome, removes Mn^{2+} from the organelle. Thus, the Mn^{2+} depletion of the phagosome microenvironment by the *Nramp* gene product may be a rate-limiting step in the metalloenzyme's production by the engulfed bacteria. This will restrict the mycobacterial ability to produce active enzymes such as SOD and prevent propagation of the ingested microorganisms. Conversely, an increased concentration of Mn^{2+} in the phagosome caused by a defective Nramp transporter (Bcg^s) may promote growth of the mycobacteria and render the organism sensitive to the pathogen. The discovery of a homologous gene in *M. leprae* supports our suggestion that Nramp might be a Mn^{2+} or other metal ion transporter. It is reasonable that the bacteria competes with the eukaryotic cell for a nutrient (metal ion) rather than a poisonous substance such as NO_2^-. Our suggestion for the possible function of Nramp and malvolio proteins as metal ion transporters can be tested in several experimental systems: (*i*) The proteins can be expressed in *Xenopus* oocytes and assayed for their transport activity. (*ii*) To assay the effect of metal ions in the growth medium to cure the phenotype of the *Drosophila* (*malvolio*) mutant. (*iii*) Expression of mouse Nramp in yeast *smf1* null mutants. The latter could provide an excellent system for identifying the function and specificity of the mammalian and insect transporters. The ramification of developing such expression systems may eventually lead to a design of new drugs against mycobacterial infections.

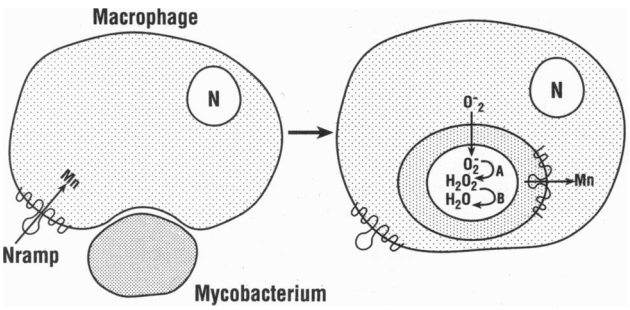

FIG. 8. A proposed role of Nramp in macrophage–pathogen interaction. Nramp is proposed to function in Mn^{2+} uptake by the macrophage and, after formation of the phagosome, to deplete its lumen of Mn^{2+}. Directionality of flow of Mn^{2+} and O_2^- is indicated by arrows. N, macrophage nucleus; A, superoxide dismutase; B, catalase.

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1. Lesuisse, E., Raguzzi, F. & Crichton, R. R. (1987) *J. Gen. Microbiol.* **133,** 3229–3236.
2. Rudolph, H. K., Antebi, A., Fink, G. R., Buckley, C. M., Dorman, T. E., Le Vitre, J., Davidow, L. S., Mao, J. I. & Moir, D. T. (1989) *Cell* **58,** 133–145.
3. Cunningham, K. W. & Fink, G. R. (1994) *J. Cell Biol.* **124,** 351–363.
4. Ghislain, M., Goffeau, A., Halachmi, D. & Eilam, Y. (1990) *J. Biol. Chem.* **265,** 18400–18407.
5. Herchuelz, A., Sener, A. & Malaisse, W. J. (1980) *J. Membr. Biol.* **57,** 1–12.
6. Crompton, M., Moser, R., Ludi, H. & Carafoli, E. (1978) *Eur. J. Biochem.* **82,** 25–31.
7. Ohsumi, Y. & Anraku, Y. (1983) *J. Biol. Chem.* **258,** 5614–5617.
8. Rooney, E. K. & Gross, J. D. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 8025–8029.
9. Schmidt, B., Wachter, E., Sebald, W. & Neupert, W. (1984) *Eur. J. Biochem.* **144,** 581–588.
10. Coleman, J. E. (1992) *Annu. Rev. Biochem.* **61,** 897–946.
11. Askwith, C., Eide, D., Ho, A. V., Bernard, P. S., Li, L., Davis-Kaplan, S., Sipe, D. M. & Kaplan, J. (1994) *Cell* **76,** 403–410.
12. Dancis, A., Yuan, D. S., Haile, D., Askwith, C., Eide, D., Moehle, C., Kaplan, J. & Klausner, R. D. (1994) *Cell* **76,** 393–402.
13. Noumi, T., Beltrán, C., Nelson, H. & Nelson, N. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 1938–1942.
14. Lawrence, C. W. (1991) *Methods Enzymol.* **194,** 273–280.
15. Sherman, F. & Hicks, J. (1991) *Methods Enzymol.* **194,** 21–37.
16. Ito, H., Fukada, Y., Murata, K. & Kimura, A. (1983) *J. Bacteriol.* **153,** 163–168.
17. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1984) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 88–148.
18. Strathern, J. N. & Higgins, D. R. (1991) *Methods Enzymol.* **194,** 319–328.
19. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
20. Rothstein, R. (1991) *Methods Enzymol.* **194,** 281–301.
21. Uchida, E., Ohsumi, Y. & Anraku, Y. (1985) *J. Biol. Chem.* **260,** 1090–1095.
22. Goffeau, A. & Dufuor, J.-P. (1988) *Methods Enzymol.* **157,** 528–532.
23. Serrano, R. (1988) *Methods Enzymol.* **157,** 533–543.
24. Yaffe, M. P. (1991) *Methods Enzymol.* **194,** 627–643.
25. Nelson, N. (1991) *Methods Enzymol.* **97,** 510–513.
26. Sherman, F. (1991) *Methods Enzymol.* **194,** 3–20.
27. White, C. & Gadd, G. M. (1987) *J. Gen. Microbiol.* **133,** 727–737.
28. Norris, P. R. & Kelly, D. P. (1977) *J. Gen. Microbiol.* **99,** 317–324.
29. Hawlitschek, G., Schneider, H., Schmidt, B., Tropschug, M., Hartl, F.-U. & Neupert, W. (1988) *Cell* **53,** 795–806.
30. West, A. H., Clark, D. J., Martin, J., Neupert, W., Hartl, F.-U. & Horwich, A. L. (1992) *J. Biol. Chem.* **267,** 24625–24633.
31. Goud, B., Salminen, A., Walworth, N. C. & Novick, P. J. (1988) *Cell* **53,** 753–768.
32. Pollock, R. A., Hartl, F.-U., Cheng, M. Y., Ostermann, J., Horwich, A. & Neupert, W. (1988) *EMBO J.* **7,** 3493–3500.
33. Yang, M., Jensen, R. E., Yaffe, M. P., Oppliger, W. & Schatz, G. (1988) *EMBO J.* **7,** 3857–3862.
34. Witte, C., Jensen, R. E., Yaffe, M. P. & Schatz, G. (1988) *EMBO J.* **7,** 1439–1447.
35. Vidal, S. M., Malo, D., Vogan, K., Skamene, E. & Gros, P. (1993) *Cell* **73,** 469–485.
36. Kishi, F. (1994) *Biochem. Biophys. Res. Commun.* **204,** 1074–1080.
37. Cellier, M. F., Govoni, G., Vidal, S., Kwan, T., Groulx, N., Liu, J., Sanchez, F., Skamene, E., Schurr, E. & Gros, P. (1994) *J. Exp. Med.* **180,** 1741–1752.
38. Barton, C. H. (1994) *J. Exp. Med.* **179,** 1683–1687.
39. Rodrigues, V., Cheah, P. Y., Ray, K. & Chia, W. (1995) *EMBO J.* **14,** 3007–3020.
40. Halbrook, J. & Hoekstra, M. F. (1995) *Mol. Cell. Biol.* **14,** 8037–8050.
41. Plant, J. E. & Glynn, A. (1976) *J. Infect. Dis.* **133,** 72–78.
42. Bradley, D. J. (1977) *Clin. Exp. Immunol.* **30,** 130–140.
43. Walker, L. & Lowrie, D. B. (1981) *Nature (London)* **293,** 69–70.
44. Flesch, E. A. & Kaufmann, S. H. E. (1991) *Infect. Immun.* **59,** 3213–3218.
45. Welch, D. F., Sword, C. P., Brehm, S. & Dusanic, D. (1979) *Infect. Immun.* **23,** 863–872.