Functional Complementation of the Yeast Divalent Cation Transporter Family SMF by NRAMP2, a Member of the Mammalian Natural Resistance-associated Macrophage Protein Family

(Received for publication, July 29, 1997, and in revised form, September 7, 1997)

Elhanan Pinner‡, Samantha Gruenheid§‡, Martine Raymond¶‡, and Philippe Gros∥

From the ‡Department of Biochemistry, McGill University, Montreal, Quebec H3G 1Y6, Canada and the ¶Clinical Research Institute of Montreal, Montreal, Quebec H2W 1R7, Canada

The mammalian NRAMP gene family has two members, NRAMP1 and NRAMP2 that encode integral membrane proteins. Nramp1 is expressed exclusively in macrophages where it is found in the phagosomal membrane, and NRAMP1 mutations cause susceptibility to infection by abrogating the capacity of macrophages to control intracellular microbial replication. Nramp2 is highly similar to Nramp1, but is expressed in several tissues and cell types. The Nramp protein family is remarkably conserved throughout evolution, and recent data suggest that the mammalian Nramp2 and the yeast homologues Smf1 and Smf2 transport divalent cations. We tested whether structural similarity between the mammalian Nramp and the yeast Smf proteins results in functional complementation in yeast. Wild-type and mutant variants of the Nramp1 and Nramp2 proteins were expressed in a yeast mutant bearing null alleles at the SMF1 and SMF2 loci, and complementation of the phenotypes of this yeast mutant was investigated. Nramp2, but not Nramp1, was found to complement hypersensitivity to EGTA of the smf1/smf2 double mutant under oxidative stress conditions (methyl viologen). We also observed that the smf1/smf2 double mutant is hypersensitive to growth at alkaline pH (pH 7.9) and that Nramp2 could complement this phenotype as well. Complementation by Nramp2 was specific and required a functional protein as independent mutations in residues highly conserved in all members of the Nramp family abrogated Nramp2 complementation. Since Mn$^{2+}$ was the only divalent cation capable of completely suppressing both the EGTA and pH phenotypes, our results suggest that Nramp2 can transport Mn$^{2+}$ in yeast.

Bcg/Lsh/Ity is a mouse locus that controls resistance to infection in vivo with Mycobacterium, Salmonella, and Leishmania (1–3). Experiments in vivo (4) and in vitro with primary cells (5–7) have shown that Bcg is expressed by macrophages and controls the ability of these cells to restrict intracellular microbial proliferation. The pleiotropic effects of Bcg on infection with unrelated microorganisms suggest a key role in natural defenses of phagocytes. Positional cloning of the Bcg locus identified the NRAMP1 gene (Natural Resistance Associated Macrophage Protein 1 (8)). The NRAMP1 mRNA is expressed almost exclusively in phagocytic cells (8), and it encodes a highly hydrophobic polytopic membrane protein composed of 12 predicted transmembrane (TM)$^i$ segments, a glycosylated extracellular loop and a consensus “transport signature” found in several prokaryotic and eukaryotic transport proteins (9). All Bcg$^i$ inbred strains show a single G169D substitution in predicted TM4 of Nramp1 (8, 10); further experiments in transgenic animals have demonstrated that NRAMP1 and Bcg/Lsh/Ity are indeed the same gene (11, 12). The Nramp1 protein is an integral membrane protein of 100 kDa that is extensively modified in mature macrophages by N-linked glycosylation (contributing approximately 50% of molecular mass) and phosphorylation (13). Subcellular localization by confocal microscopy indicated that Nramp1 is located in the late endosomal compartment (Lamp1-positive) of macrophages (14). Upon phagocytosis, Nramp1 is recruited to the phagosomal membrane and remains associated with this structure during its maturation to phagolysosome (14). Nramp1 may, in an unknown fashion, modulate the content of this vesicular compartment to affect microbial replication.

We have identified a second NRAMP gene in mouse and humans, NRAMP2. The Nramp1 and Nramp2 proteins are highly similar (77% overall similarity), with identical hydropathy profiles and predicted secondary structures. As opposed to its phagocytic-specific NRAMP1 counterpart, both the human and mouse NRAMP2 mRNAs are expressed in several tissues and cell types analyzed (15). Recent studies by electrophysiology have indicated that Nramp2 can function as a broad specificity divalent cation transport system (16), while mice with a mutant NRAMP2 gene are deficient in intestinal iron uptake (17). Distant NRAMP homologues have also been identified in the fly Drosophila melanogaster, the worm Caenorhabditis elegans, the plant Oriza sativa, the yeast Saccharomyces cerevisiae, and even in bacteria such as Escherichia coli$^2$ and Mycobacterium leprae (18). Optimal amino acid sequence alignment reveals remarkable sequence identities of 39% (bacterium), 41% (yeast), 60% (plant), 67% (worm), and 70% (fly) with the mammalian proteins, over their common region of overlap. This family is defined by the presence of a highly conserved hydrophobic core encoding 10 TM segments, which includes several invariant charged residues and the transport signature (8) on the cytosolic side of the membrane (TM5–9 interval, Ref. 18).

1 The abbreviations used are: TM, transmembrane; SD, synthetic medium; SD–Ura, synthetic medium lacking uracil; Mes, 4-morpholineethanesulfonic acid; PCR, polymerase chain reaction; $T_c$, temperature-sensitive.

2 M. Cellier and P. Gros, unpublished data.

* Supported by National Institutes of Health Grant AI35237–04A1 (to P. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by a studentship from the Medical Research Council of Canada.

§ International Research Scholar of the Howard Hughes Medical Institute and to whom correspondence should be addressed: Dept. of Biochemistry, McGill University, 3655 Drummond, Montreal, Quebec H3G 1Y6, Canada. Tel.: 514-398-7291; Fax: 514-398-2603; E-mail: gros@medcor.mcgill.ca.

This paper is available online at http://www.jbc.org
The two yeast homologues, SMF1 and SMF2, were cloned by their ability to suppress misI, a T₄ mutation which affects a Mn²⁺-dependent signal peptidase (PEP1) essential for import of cytosolic proteins into mitochondria (19). The double smf1/smf2 mutant shows a slow growth phenotype and is impaired in import and processing of mitochondrial proteins (19). Recently, SMF1 was re-isolated by Supek et al. as an extrageneic high copy suppressor of a phenotype of hypersensitivity to the chelating agent EGTA (20). These authors presented evidence suggesting that Smf1 may be a Mn²⁺ transporter, including the observations that SMF1 inactivation causes hypersensitivity to EGTA, and that cells overexpressing Smf1 show increased accumulation of ⁵⁴Mn²⁺ (20). Recent data have suggested that the yeast SMF2 gene may similarly encode a divergent cation transporter (21). In this study, we set out to determine if the sequence similarity noted between mammalian Nramp and yeast Smf proteins translates into functional homology. We have attempted to complement the phenotypic characteristics of a double smf1/smf2 yeast mutant by expressing in this strain the mammalian Nramp1 and Nramp2 proteins.

**MATERIALS AND METHODS**

**Yeast Strains—** *S. cerevisiae* strain SMF1/2KO, thereafter strain SMF1/2, is a mutant in which the SMF1 and SMF2 genes have been inactivated (MATa ura3–52 leu2–3–112 gal2 SMF1::LEU2, SMF2::LEU2, 19) and was obtained from A. L. Horwich (Department of Genetics, Yale University).

**Culture Media—** For selection of transformants and mutants, cells were grown on synthetic medium (SD) containing 0.67% yeast nitrogen base, 2% dextrose, and a mixture of amino acids and nucleotides but lacking uracil (SD–Ura, see Ref. 22). For functional complementation of the smf1/smf2 mutant on EGTA solid medium, cells were grown on YPD (2% bacto-peptone, 1% yeast extract, 2% dextrose) supplemented with 50 mM Na-Mes (pH 6.5) and 0.5 mM DTT (Sigma) and various concentrations of EGTA and incubated for 3 days at 30 °C. For experiments in liquid medium, cells were seeded in 96-well plates (100 µl/well), and growth over time (30 h) was monitored by measuring optical density at 595 nm using an ELISA microplate reader (Bio-Rad model 450). For functional complementation of the smf1/smf2 mutant on alkaline solid medium, cells were plated on YPD-agar supplemented with 0.67% yeast nitrogen base, 2% dextrose, and a mixture of amino acids and nucleotides but lacking uracil (SD–Ura, see Ref. 22). For selection of transformants and mutants, cells were plated on YPD-agar supplemented with 0.67% yeast nitrogen base, 2% dextrose, and a mixture of amino acids and nucleotides but lacking uracil (SD–Ura, see Ref. 22).

**RESULTS**

Expression of Mouse Nramp1 and Nramp2 in Yeast—To determine the degree of functional homology between the mammalian Nramp and the yeast Smf proteins, we set out to test whether expression of mammalian Nramp1 or Nramp2 in a double smf1/smf2 null mutant (*S. cerevisiae* strain SMF1/2, 19) would correct phenotypes associated with inactivation of SMF genes. We have previously obtained high level expression of the mouse Nramp1 protein in yeast using a modified cDNA in which the 5'-untranslated region was deleted and replaced by the hexanucleotide TAAAAA, and 4 c-Myc epitope tags were fused in-frame at the C terminus of the protein. The cDNA was cloned behind the strong ADH1 promoter of the vector pVT (pVT-Nr1; formerly pVT-yNr1Myc4, Ref. 13). Similar alterations were introduced in NRAMP2 and SMF1 prior to cloning into pVT to produce pVT-Nr2 (2 c-Myc tags), pVT-SMF1 (2 c-Myc tags, see “Materials and Methods”). Plasmids were introduced in SMF1/2, mass populations of transformants were selected on SD-Ura, and the level of expression of the three recombinant proteins was monitored in enriched membrane fractions of Ura⁻ cells by immunoblottting with the anti-c-Myc epitope antibody 9E10 (Ref. 24, Fig. 1). A specific immunoreactive band of 60–65 kDa approximate molecular mass was detected in pVT-Nr1 (lane 2), pVT-Nr2 (lane 3), and pVT-SMF1 (lane 4) transformants but was absent from control cells transformed with the pVT vector (lane 1). Detected protein expression levels were highest for Nramp1, lowest for Nramp2, and intermediate for Smf1. A specific immunoreactive band of apparent electrophoretic mobility ~140 kDa was noted in pVT-Nr2 but not in pVT-SMF1 cells. Although the origin of this band remains unknown, it has been seen for Nramp1 expressed in another yeast strain (13), and it may result from nonspecific aggregation of the protein prior to or during electrophoresis. Thus, results in Fig. 1 show stable expression of mammalian Nramp1 and Nramp2 in the membrane fraction of yeast cells.

**Nrpamp2 Complements EGTA Sensitivity of an smf1/smf2 Null Mutant**

**Figure 1** shows stable expression of mammalian Nramp1 and Nramp2 in the membrane fraction of yeast cells.
Double Mutant—Smf1 has been proposed to function as a Mn2⁺ transporter, and yeast strains carrying a null allele at smf1 cannot grow on medium containing 12.5 mM EGTA (20). We tested the ability of mammalian Nramp1 and Nramp2 to complement this phenotype. To eliminate possible interference of other SMF genes in this assay, we used a yeast tester strain in which both SMF1 and SMF2 were inactivated by targeting (SMF1/2). In preliminary experiments, it was noted that imposing oxidative stress by adding 0.5 mM methyl viologen (paraquat) to the growth medium significantly increased the EGTA sensitivity of SMF1/2 to submillimolar concentrations (Fig. 2A; pVT control, IC₅₀ = 130 μM). Under such culture conditions, Smf1 expression in SMF1/2 conferred a 20-fold elevation of cellular resistance to EGTA (Fig. 2A; Smf1, IC₅₀ = 2.7 mM) over background levels seen in the pVT control (IC₅₀ = 130 μM).

We observed that Nramp2 could complement EGTA sensitivity of SMF1/2 (IC₅₀ = 930 μM) restoring growth in EGTA at a level intermediate (8 × resistance) between that observed in pVT-SMF1 transformants and that of the pVT controls (Fig. 2A). In contrast to Nramp2, transformation with the Nramp1-expressing plasmid (pVT-Nr1) did not increase cellular resistance to EGTA, with pVT-Nr1 transformants showing dose-response kinetics similar to the pVT control (Fig. 2A). These results indicate that, like Smf1, mammalian Nramp2 can functionally complement hypersensitivity to EGTA in a yeast mutant lacking functional Smf1 and Smf2 transporters.

To determine which of the various divalent metals chelated by EGTA were implicated in growth inhibition observed for pVT and Nramp1 transformants of the SMF1/2 strain, we examined the effect of increasing concentration of different metals on the IC₅₀ for EGTA of the various yeast transformants (Fig. 2B). We observed that the addition to the growth medium of Mn²⁺ salts produced a clear, concentration-dependent increase in the EGTA IC₅₀ (Fig. 2B). This effect was most obvious for the pVT and Nramp1 transformants, and at 100 μM Mn²⁺, the effect was optimal and all strains had a similar IC₅₀ for EGTA. The addition of other metals such as Cu²⁺, Fe²⁺, and Zn²⁺ to the growth medium at 100 μM did not completely suppress the EGTA inhibition (not shown). These results suggest that chelation of Mn²⁺ by EGTA is most likely responsible for the growth inhibition phenotype of the SMF1/2 mutant that is complemented by Nramp2.

Independent Mutations in Nramp2 Abrogate EGTA Complementation—To determine if complementation of EGTA hypersensitivity in the SMF1/2 mutant by Nramp2 is specific and requires a functional protein, independent mutations were introduced in Nramp2 (Fig. 2C), and the ability of these mutants to complement growth in EGTA was tested in dose-response experiments (Fig. 2D). Three independent mutations were introduced in the transport signature located in the fourth intracellular loop of the protein delineated by TM8 and TM9. This motif 1) is the region of highest sequence conservation between the mammalian and yeast proteins (See “Discussion” and Ref. 18), 2) is related to the EAA box of bacterial periplasmic permeases (8), and 3) is essential for activity as mutations introduced in this motif abrogate function of bacterial transporters (25). Glutamines 384 and 395, and glycine 394, which are conserved in all the members of the Nramp family (Fig. 2C), were replaced. The glutamines were changed to structurally similar but charged glutamate (Q384E, Q395E), while the glycine was substituted to valine (G394V), a bulkier residue. Immunoblotting of membrane fraction of yeast populations transformed with these mutants indicated that the level of expression of the three Nramp2 variants was similar to that seen in yeast cells expressing wild-type Nramp2 (Fig. 1, compare lanes 5–7 to lane 3). When tested for growth in medium containing increasing amounts of EGTA (Fig. 2D), the Q384E and G394V mutants showed IC₅₀ values similar to that of pVT control cells, indicating complete loss of Nramp2 function. On the other hand, the Q395E Nramp2 mutant conferred a small but reproducible increase in cellular resistance to EGTA (Fig. 2C; IC₅₀ = 370 μM; 3 × resistance) above levels seen in pVT controls. These results indicate that complementation of EGTA hypersensitivity of the SMF1/2 mutant by Nramp2 is specific, most likely requires an intact protein, and suggests that the highly conserved Gln-384 and Gly-394 residues in the transport signature are essential for Nramp2 function.

Nramp2 Complements Alkaline pH Sensitivity of an smf1/ smf2 Double Mutant—While analyzing the phenotypic characteristics of the SMF1/2 mutant, we noted that its growth at alkaline pH was impaired. When plated on YPD-agar supplemented with 50 mM Tris-Cl, pH 8.5 (final pH, 7.8–7.9, “YPD7.9”), neither SMF1/2 cells nor pVT transformants grew on this medium even after prolonged incubation periods (Fig. 3A, panel 1). Transformation of SMF1/2 with pVT-SMF1 restored growth on alkaline medium (Fig. 3A, panel 2), indicating that this phenotype is indeed linked to the absence of functional Smf protein in SMF1/2 mutant. Similar to the effect on EGTA hypersensitivity, wild-type Nramp2 could restore growth of SMF1/2 at alkaline pH (Fig. 3A, panel 4) while Nramp1 was unable to do so (Fig. 3A, panel 3). Also in agreement with results of complementation of EGTA sensitivity, the Q384E and G394V Nramp2 mutants were inactive (panels 6 and 7), while the Q395E expressing mutant (panel 8) grew at alkaline pH with characteristics similar to that seen for wild-type Nramp2 (panels 4 and 5). These results show that Nramp2 can complement another phenotypic characteristic of the SMF1/2 mutant and also suggest that hypersensitivity to EGTA and alkaline pH are caused in SMF1/2 by the same molecular event, which may reflect a mechanistic basis common to Nramp2 and Smf1.

To further probe the relationship between alkaline pH sensitivity and divalent metal availability, the various SMF1/2 transformants were grown in YPD7.9 containing increasing concentrations of different metals, and the effect of the metal ions on growth was monitored (Fig. 3B). Mn²⁺ was the most efficient cation for suppressing sensitivity to alkaline pH in both pVT and pVT-Nr1 transformants with a half-maximal growth obtained with 1 μM MnCl₂ (Fig. 3B). A moderate but consistent stimulation of growth by Mn²⁺ ions was also noted.

**Figure 1. Expression of mammalian Nramp proteins in yeast.** Saccharomyces cerevisiae strain SMF1/2 was transformed with the pVT expression plasmid alone (lane 1) or pVT containing full-length cDNAs for either SMF1 (lane 4), wild-type mouse NRAMP1 (lane 2) or NRAMP2 (lane 3), or mutant variants of NRAMP2 Q384E (lane 5), G394V (lane 6), or Q395E (lane 7). All proteins were tagged by the in-frame addition at their C terminus of a short antigenic epitope corresponding to the c-Myc protein. Enriched membrane fractions were prepared from pools of yeast transformants, separated by 12% SDS-polyacrylamide gel electrophoresis, and analyzed by immunoblotting with the anti-c-Myc epitope monoclonal antibody 9E10 and a secondary anti-mouse antibody conjugated to horseradish peroxidase. The positions of reference molecular mass markers are indicated to the left of the immunoblot.
in pVT-Nr2 and pVT-SMF1 transformants at higher MnCl₂ concentrations (>1 mM; Fig. 3B). Fe²⁺ ions were less effective than Mn²⁺ ions although FeCl₂ at 1 mM completely restored growth at alkaline pH with a half-maximal growth obtained of 100 and 300 μM for pVT and pVT-Nr1, respectively (Fig. 3B). CuSO₄, ZnSO₄, and CoCl₂ were even less active than FeCl₂ at suppressing growth inhibition at alkaline pH, whereas CaCl₂ had no effect at all (data not shown). These results indicate that, as for the EGTA sensitivity, Mn²⁺ ions are the most efficient at suppressing sensitivity to alkaline pH, suggesting that the two phenotypes may be linked by a common mechanistic basis.

**DISCUSSION**

An important role for the NRAMP1 gene in host defenses against intracellular infections has been demonstrated in vivo in transgenic animals bearing either a null allele (11) or a gain of function allele (12) at the NRAMP1/Bcg locus. The identification of professional phagocytes (macrophages, polymorphonuclear leukocytes) as the cell type expressing Nramp1 (8, 26), together with the subcellular localization of Nramp1 to the maturing phagosome in these cells, strongly suggest that Nramp1 controls intracellular microbial replication by altering the intravesicular composition of the microbe-containing phagosome. Unlike NRAMP1, NRAMP2 is widely expressed in most tissues tested but the subcellular localization of its protein product is unknown. While the substrate of Nramp1 in macrophages has yet to be identified, recent studies show that Nramp2 can function as a broad specificity divalent cation transporter in *Xenopus laevis* oocytes (16). This transport is pH-sensitive, electrogenic, and appears to be coupled to proton movement (16). Finally, mice with mutated NRAMP2 are deficient in intestinal iron uptake (17).

The NRAMP family is highly conserved in evolution and has three members in yeast, known as SMF1, SMF2, and SMF3 (reviewed by Cellier et al., 18). The sequence identity between the human and yeast proteins reaches 41% in the highly hydrophobic core consisting of the first ten N-terminal TM segments of the protein, with several of these domains highly conserved in human and yeast proteins. Interestingly, Smf1 was recently shown to be a Mn²⁺ transporter (20). To monitor the extent of functional homology between the yeast and mammalian proteins, we attempted to complement a double smf1/smf2 null mutant (SMF1/2) by either Nramp1 or Nramp2 using two phenotypic markers. The first one is the hypersensitivity of SMF1/2 cells to EGTA, which is exacerbated under oxidative stress induced by methyl viologen. This phenotype is consistent with a key role of transition metals transported by Smf proteins as cofactors for detoxifying enzymes such as the superoxide dismutases Sod1 (Cu²⁺, Zn²⁺) and Sod2, (Mn²⁺) essential for survival under oxidative stress (27, 28).
Complementation of Yeast SMF Genes by NRAMP2

Fig. 3. Complementation of the phenotype of hypersensitivity to alkaline pH of the SMF1/2 yeast mutant by mammalian Nramp2. A, similar numbers of SMF1/2 yeast cells transformed with the pVT expression plasmid alone (1) or pVT containing either SMF1 (2), wild-type mouse NRAMP1 (3) or NRAMP2 (4, 5), or mutant variants of NRAMP2 Q384E (7), G394V (6), or Q395E (8) were plated on YPD-agar solid medium containing 50 mM Tris-Cl (final pH 7.8–7.9), grown for 72 h at 30°C, and photographed. B, the curvative effect of increasing concentrations of Mn²⁺ or Fe³⁺ (dose response) on the sensitivity to alkaline pH of each population of SMF1/2 transformants was measured in liquid YPD7.9. The relative growth of each population in each condition is expressed as a percentage of maximal growth observed in the presence of an optimal metal concentration. The maximal growth for the different transformants was similar. Yeast transformants expressing either control pVT plasmid (●) and wild-type SMF1 (○), NRAMP1 (■), or NRAMP2 (▲) are identified.

We analyzed the observed inability of SMF1/2 to grow at pH 7.9. Although a common biochemical basis for the hypersensitivity to EGTA and alkaline pH in SMF1/2 can only be speculated upon (see below), both phenotypes can be suppressed by Mn²⁺, suggesting that both effects are specific and are related to the loss of SMF gene function in this mutant. We observed that expression of Nrramp2 in SMF1/2 could correct both hypersensitivity phenotypes at a level comparable with that seen in SMF1 transformants, suggesting that structural similarity in this family underlies a common transport mechanism. In our experimental setting, Mn²⁺ was the only metal capable of efficiently suppressing both EGTA and alkaline pH sensitivity, and as SMF1 was shown to be a Mn²⁺ transporter, our results suggest that Nrramp2 may also transport Mn²⁺ in yeast. The poor level of suppression of alkaline pH hypersensitivity with Fe³⁺ in our experimental conditions is somewhat surprising in view of the proposed role of Nrramp2 in iron transport (16, 17). One possible explanation is that at the alkaline pH (8.0) used to monitor functional complementation, a significant portion of iron in the medium may be in the Fe³⁺ form, which may not be a substrate for Nrramp2.

Unlike Nrramp2, Nrramp1 did not complement the SMF1/2 hypersensitivity phenotypes. This different activity of the two mammalian proteins in these assays could be explained by either functional differences such as non-overlapping substrate specificity or by the inability of Nrramp1 to function in the yeast cellular environment, including targeting to a different subcellular compartment. Nevertheless, the strong homology between Nramp1 and Nramp2 raises the possibility that Nramp1 may also transport divalent cations.

The observation that SMF1/2 cells are hypersensitive to alkaline pH is intriguing and may suggest that transition metals are necessary for certain yeast enzymes that are essential for survival under alkaline conditions. For example, it is known that yeast mutated in the vacuolar H⁺-ATPase fail to acidify the vacuole and do not grow at alkaline pH (29). A direct or indirect role of divalent cations such as Mn²⁺ in vacuolar acidification could underlie the alkaline sensitivity phenotype. Alternatively, Mn²⁺ transport by Smf1 and Nrramp2 proteins may be coupled to proton movement, as recently suggested (16). Suppression of the pH sensitivity phenotype by Mn²⁺ in control cells that lack either SMF1 or SMF2 would argue against such a possibility, unless the suppressor effect of Mn²⁺ in these cells involves another functionally related transporter, for example the SMF3 gene for which there is no known function.

The complementation by Nrramp2 of the SMF1/2 mutant was specific and was abrogated by 2 out of 3 mutations in a protein segment which is highly conserved in members of the Nrramp family (Fig. 2C). This 20-residue segment (positions 367–387), predicted to overlap part of TM8 and the intracellular loop delineated by TM8 and TM9, shares similarity with three sets of residues: 1) the sequence motif known as the “binding protein-dependent transport system inner membrane component signature” (transport signature, Ref. 8) ((E, Q,X)₅(T, A)₂(G/X)₅(L, I, V, M, Y, F, A)X₄(F, L, I, V) (P, K)), also known as the EAA box (31); 2) TM3 of the prototrophic subunit of the vacuolar H⁺-ATPase family (periodical distribution of Gly and other small residues, positions 366, 368, 369, 371, 373, 376, 379, and 380 in Nrramp1 protein sequence, see Ref. 32); and 3) the permeation pore of the shaker type K⁺ channels (TM1-X₄-G-(D/Q)-X₄-GF; positions 373–387, 33). In each of these families, the conserved residues map to a region shown to be essential to function (25, 35, 36). Two of the mutations introduced, Q384E and G394V, abolished Nrramp2 function, indicating that these two residues are important for the transport activity common to Nrramp2 and Smf1 proteins. Glutamine 384 is the first residue of the transport signature and in some eukaryotic and prokaryotic members of this family, a glutamate can be found at this position. Our results show that such a substitution cannot be tolerated in Nrramp2 without loss of function. Likewise, the Gly-394 residue is absolutely conserved in all members of the Nrramp family (Fig. 2C) and in the permeation path of K⁺ channels. The loss of function noted in the Nrramp2 G394V mutant suggests that this flexible residue is essential for a structural or functional characteristic common to both of these gene families. The highly conserved Gin-395 does not appear essential for function as measured in our assay since introduction of an additional negative charge at that site is without effect on complementation.

In conclusion, the observed complementation between mammalian Nrramp2 and yeast Smf proteins suggests that this protein family is comprised of divalent cation transporters that have been structurally and functionally conserved during evolution. In addition, complementation by the EGTA and alkaline pH hypersensitivity phenotypes of the SMF1/2 mutant provides a functional assay for the structure-function analyses of Nrramp2 in yeast.

Acknowledgment—We are indebted to Dr. A. Horwich (Yale University) for the gift of the yeast smf1/sm2 mutant strain.

REFERENCES
1. Gros, P., Skamene, E., and Forget, A. (1981) J. Immunol. 127, 2417–2421
2. Plant, J., and Glynn, A. A. (1976) J. Infect. Dis. 133, 72–78
3. Bradley, D. J. (1977) Clin. Exp. Immunol. 30, 130–140
4. Gros, P., Skamene, E., and Forget, A. (1983) J. Immunol. 131, 1966–1972
5. Stach, J. L., Gros, P., Forget, A., and Skamene, E. (1984) J. Immunol. 132, 888–892
6. Lissner, C. R., Swanson, R. N., and O’Brien, A. D. (1983) J. Immunol. 131, 3006–3013
7. Creeker, P. R., Blackwell, J. M., and Bradley, D. J. (1984) Infect. Immun. 43, 1033–1040
8. Vidal, S. M., Malo, D., Vogan, K., Skamene, E., and Gros, P. (1993) Cell 73, 469–485
