Evolution of Differences in Transport Function in Slc11a Family Members

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Slc11a1 (formerly Nramp1) is a proton/divalent cation transporter that regulates cation homeostasis in macrophages. Slc11a2 mediates divalent cation uptake via the gut and delivery into cells. The mode of action of the two transporters remains controversial. Heterologous expression in frog oocytes shows Slc11a2 is a symporter, whereas Slc11a1 is an antiporter fluxing divalent cations against the proton gradient. This explains why Slc11a2, but not Slc11a1, can complement EGTA sensitivity in smf1Δ/smff2Δ/smff3Δ yeast. However, some studies of transport in mammalian cells suggest Slc11a1 is a symporter. We now demonstrate that Slc11a1, but not Slc11a2, complements a divalent cation stress phenotype in bsd2Δ/rer1Δ yeast. This is the first description of a yeast complementation assay for Slc11a1 function. Given the prior demonstration in frog oocytes that Slc11a1 acts as an antiporter, the most plausible interpretation of the data is that Slc11a1 is rescuing bsd2Δ/rer1Δ yeast by exporting divalent cations. Chimaeras define the N terminus, and a segment of the protein core preceding transmembrane domain 9 through transmembrane domain 12, as important in rescuing the divalent cation stress phenotype in yeast expressing Slc11a1 orthologues show that symporter activity is ancestral. Molecular changes that mediate rescue of the divalent cation stress phenotype post-date frogs and co-evolved with Slc11a1 orthologues that regulate divalent cation homeostasis in macrophages and resistance to infection in chickens and mammals.

Slc11a1 (formerly Ity/Lsh/Bcg/Nramp1) is a proton/divalent cation transporter with 12 putative transmembrane domains (TMD) (1) that localizes to late endosomes and lysosomes of macrophages (2, 3) and dendritic cells (4), and tertiary granules of neutrophils (5). It is recruited to phagosome membranes in macrophages infected with Mycobacterium (2, 3) or Leishmania (3), and regulates cellular divalent cation homeostasis (6, 7). Polymorphisms at murine Slc11a1 and human SLC11A1 contribute to susceptibility to numerous infectious and autoimmune diseases (8, 9). Slc11a2 (also Nramp2/DMT1/DCT1) shares 64% amino acid identity with Slc11a1 (10) and regulates divalent cation uptake via the gut and delivery of iron into multiple cell types (11, 12). Different isoforms can be distinguished by different C-terminal amino acid sequences, and by the presence or absence of an iron response element located in the 3′-untranslated region of the mRNA (13). The IRE-containing isoform (Slc11a2-IRE) is expressed predominantly in epithelial cells and localizes to late endosome/lysosomes, whereas the non-IRE-containing isoform (Slc11a2-nonIRE) is expressed in blood cell lineages and localizes to early endosomes (13). Both isoforms localize to their respective endosomal compartments and to apical membranes when expressed in polarized Madin-Darby canine kidney cells (13). Mutation at Slc11a2 in mice and rats is associated with microcytic anemia (14, 15).

Fe2+, Zn2+, Mn2+, Co2+, Cd2+, Cu2+, Ni2+, and Pb2+ have all been shown to evoke currents in voltage-clamped frog oocytes heterologously expressing Slc11a2 (16), but there is discussion as to whether all act as substrates for Slc11a2 (17). Slc11a1 may transport an equivalent range of divalent cations, but so far only Fe2+, Zn2+, and Mn2+ transport has been measured following heterologous expression in frog oocytes (18). These studies in frog oocytes demonstrate that, whereas Slc11a2 is a symporter that transports divalent cations and protons with the proton gradient (16), Slc11a1 is an antiporter that fluxes divalent cations in either direction against the proton gradient (18). This provides an explanation for data showing that murine/human Slc11a2/SLC11A2, but not Slc11a1/SLC11A1, can complement EGTA and pH sensitivity phenotypes in yeast deleted for the Slc11a1 homologues SMF1/2/3, even though both were equivalently expressed at the yeast plasma membrane (19, 20).

It follows from the frog oocyte studies that Slc11a2 would transport divalent cations with the proton gradient from acidic intracellular vesicles of mammalian cells to the cytoplasm. This is confirmed by studies showing that Slc11a2 and the transferrin receptor colocalize in acidic endosomes and are functionally coupled to effect pH-dependent iron uptake across the endosomal membrane (21). In contrast, Slc11a1 would be predicted to flux divalent cations from the cytoplasm into the...
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Yeast Strains and Plasmids—Yeast strains were derivatives of S. cerevisiae MSY6210 or MSY6211 (32) with SMF1, SMF2, and/or SMF3 genes disrupted by transformation with smf1Δ::HIS3, smf2Δ::KANΔ, or smf3Δ::LEU2 linear PCR products, respectively. The smf1Δ knock-out strain was mated with the smf3Δ strain and diploids selected on –ade, –lys minimal medium, sporulated, and tetrads disrupted with β-glucuronidase and plated onto rich medium. Double knock-out mutants were identified by PCR of genomic DNA and the remaining double and triple mutants were generated by PCR construct-mediated gene deletion. The single and double bsd2Δ and rer1Δ strains were generated as described (31). To optimize expression of the Slc11a proteins the natural 5’-untranslated region was removed and replaced by the trinucleotide AAA, which is mediated efficient initiation of protein synthesis by the yeast translation machinery (32). C-terminal HA- or GFP-tagged Slc11a cDNAs (see below) were cloned into the YIpplac111 base vector pVJ97 (33) in which expression of the mammalian proteins is driven by the TPI promoter and PGK terminator.

Slc11a Constructs—TRiZol (Invitrogen) was used to extract total RNA from cells and tissues. Cells were grown to confluency in T75 flasks and rinsed in RPMI prior to addition of TRiZol and lysis. Tissue was homogenized in a glass Teflon homogenizer. Extraction of RNA was according to the manufacturer’s protocol. Concentration and purity of total RNA was determined using the NanoDrop-1000 spectrophotometer (version 3.1.0). Only RNA with and A260/A280 ratio 1.9–2.1 was used to make first strand cDNA. cDNA for Slc11a homologues was amplified from: malvolio (NCBI accession number U23948) from Drosophila melanogaster RNA from pooled larvae and adult (gift from David Lomas, CIMR), Oncorhynchus mykiss Slc11a-alpha (NCBI accession number AF054808) and Slc11a-beta (NCBI accession number AF054809) from cDNA constructs prepared from gill RNA (gift from Nic Bury, King’s College, London), Takifugu rubripes Slc11a1-alpha (NCBI accession number AJ496547) from yeast RNA prepared from total kidney (Geneservice, UK), Xenopus laevis Slc11a1-like homologue from cDNA IMAGE clone 7020838 (Geneservice, UK) from oocyte RNA, Gallus gallus SLC11A1 (NCBI accession number NM_204964) and SLC11A2 (NCBI accession number DQ848155) from RNA from lipopolysaccharide-stimulated macrophages (gift from Pete Kaiser, Institute for Animal Health, UK), Mus musculus Slc11a1 (NCBI accession number NM_013612), Slc11a2 without an iron response element (Slc11a2-nonIRE; NCBI accession number BC019137) and Slc11a2 with an iron response element (Slc11a2-IRE; NCBI accession number P49282) from a mouse macrophage cDNA library (34) or mouse macrophage RNA (35), and Homo sapiens SLC11A1 (NCBI accession number BC071165) and SLC11A2 (NCBI accession number BC002592) from RNA from the macrophage cell line U937 (ECCAC reference number 85011440). The 6 possible chimaeras of the N terminus, the corem and the C terminus of murine Slc11a1 and Slc11a2-nonIRE were made by PCR using the megaprimer

acidic late endosomal/lysosomal compartments against the proton gradient. Nevertheless, using an indirect method to measure intracellular Mn2+ flux across phagosomes of macrophages, Jabado et al. (22) presented evidence that Slc11a1 exetrudes Mn2+ from zymosan-loaded phagolysosomes. These authors therefore concluded that, like Slc11a2, Slc11a1 acts as a symporter transporting divalent cations out of the acidified phagosome into the cytoplasm. As a consequence, they suggest that Slc11a1 could starve intracellular pathogens of essential cations, thereby mediating resistance to infection. Contrary to this were the studies of Kuhn et al. (23, 24) that demonstrated differential transport of iron into Mycobacterium-containing phagosomes isolated from the RAW264.7 macrophage cell lines transfected with wild type Slc11a1 Gly169 or mutant Slc11a1 Asp169, and into phagosomes of intact infected macrophages. The view of these authors was that delivery of ferrous iron into the phagolysosome of infected macrophages could contribute to antimicrobial activity through production of toxic radical via the Haber-Weiss and Fenton reactions.

To understand more about its function, and to provide a simplified assay system for screening of potential inhibitors of transport function, it would be advantageous to have an assay in which the function of Slc11a1 could be measured using yeast complementation. We surmised that, if our previous observation (18) of antiporter function in frog oocytes was correct, it should be possible to develop such an assay under conditions of divalent cation stress, as opposed to the Mn2+ depletion and EGTA sensitivity generated by knock-out of the yeast Slc11a orthologues SMF1/2/3 (19, 20). To accomplish this we took advantage of the observation that, under metal replete conditions, the Bsd2 protein regulates metal homeostasis in yeast by trafficking Smf1p and Smf2p to the vacuole for degradation (25). More recent studies (26) show that Bsd2 works with the Tre1/Tre2 proteins to signal Smf1 ubiquitination by Rsp5, thereby moving Smf1 into multivesicular bodies and the vacuole. The BSD2 gene (bypassed the SOD defect) was originally prepared from total kidney (Geneservice, UK), Oncorhynchus mykiss Slc11a-alpha (NCBI accession number AJ496547) from RNA from the macrophage cell line U937 (ECCAC reference number 85011440). The 6 possible chimaeras of the N terminus, the corem and the C terminus of murine Slc11a1 and Slc11a2-nonIRE were made by PCR using the megaprimer.
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technology (36) or by using unique internal restriction enzyme sites. Chimeric constructs with the Slc11a2-IRE were not generated. The N terminus was defined as the residues from the initiating methionine to the first TMD, the core as the start of TMD 1 to end of TMD 12 with inter-joining loops, and the C terminus as the residues from the end of the TMD 12 to the STOP codon.

Yeast EGTA and Metal Assays—The smf1Δ/smff2Δ/smff3Δ and bsdΔ2/avr1Δ transformants were grown with agitation overnight in selective medium, diluted back, and harvested at early log phase. Cells were diluted to \( \sim 1 \times 10^7 \) cells/ml in medium and serial dilutions of 50 μl of cell culture and 50 μl of medium were prepared in sterile 96-well plates. For each transformant 3 μl of each dilution was pipetted onto EGTA or divalent metal cation containing YNB plates. The range of final EGTA and divalent cation concentrations was optimized in order for the various mutants to show the clearest growth-deficient phenotype. As a positive control for even growth of all strains under no selective pressure, cells were dotted in parallel onto YNB plates with no added EGTA or heavy metals. Plates were incubated at 30 °C for 3–4 days. All experiments were carried out 3–6 times using independent yeast transformants.

SDS-PAGE and Western Blotting—Transformed yeast cells were grown with agitation overnight, diluted back and harvested at early log phase. Cells were resuspended in lysis buffer (0.2 M NaOH, 0.2% 2-β-mercaptoethanol) and trichloroacetic acid was added to a final concentration of 5%. Precipitates were collected, neutralized, and dissolved in 1 M Tris base and dissociation buffer (4% SDS, 0.1 M Tris hydrochloride, pH 6.8, 4 M EDTA, 20% glycerol, 2% 2-β-mercaptoethanol, 0.02% bromphenol blue), and incubated at 37 °C for 10 min. SDS-PAGE was performed using the Bio-Rad protein II minigel system according to the manufacturer’s instructions (Hemel Hempstead, Herts, UK). For Western blotting Hybond-C nitrocellulose membranes were used (Amersham Biosciences) and membranes were probed with primary anti-GFP antibody (ab6556, polyclonal rabbit, Abcam, UK), anti-HA antibody (12CA5 Boehringer, UK), or anti-ARF1 (a gift from Reiner Duden, CIMR).

Microscopy—Yeast cells transformed with GFP-tagged constructs were grown to early log phase in selective medium and imaged in water with a Bio-Rad Radiance confocal microscope, using a single slow scan.

Phylogenetic Analysis—Multiple sequence alignments were produced using ClustalW. Based on the alignments, phylogenetic trees were constructed using quartet sampling and Neighbor-joining parameter estimation in TREE-PUZZLE, version 5.0.

Supplementary Data—Supplementary data are available at Journal of Biological Chemistry Online.

RESULTS

Slc11a2 but Not Slc11a1 Complements EGTA Sensitivity Phenotype in the Smf1Δ/2/3 Knock-out Yeast Strain—We first generated Smf knock-out yeast strains and established a clear EGTA sensitivity assay on solid medium. The double mutant smf1Δ/smff2Δ and the smf1Δ/smff2Δ/smff3Δ triple mutant showed the highest EGTA-sensitive growth deficiency (Fig. 1A, strains 6 and 9). The growth deficiency was specific to EGTA, was rescued by adding Cu²⁺ or Mn²⁺, and could be complemented by re-introducing full-length SMF2 (Fig. 1B). The smf1Δ/smff2Δ/smff3Δ strain was selected for further complementation studies.

The murine Slc11a1, Slc11a2-nonIRE, and Slc11a2-IRE genes were modified at the C terminus by in-frame addition of a GFP or a HA tag. Positive transformants were tested for protein expression by Western blotting using the anti-GFP antibody (data not shown) or the anti-HA antibody (Fig. 1C). Immuno reactive bands were absent from control cells transformed with empty HA vector (Fig. 1C, lane 1), whereas specific bands were detected in Slc11a1-HA (lane 2), Slc11a2-nonIRE-HA (lane 3), and Slc11a2-IRE-HA (lane 4) transformed cells. Expression was equivalent for all Slc11a proteins with no differences in levels of degradation between the three recombinant proteins. Live cells expressing GFP-tagged constructs were examined directly by fluorescence microscopy (Fig. 1D). As observed previously for Slc11a1 and Slc11a2 expression in fission yeast (19, 20), expression of GFP-tagged Slc11a1 and Slc11a2 in S. cerevisiae exhibited a double ring-like signal surrounding both the nucleus (as identified by comparison with the phase contrast image) and the cell surface. This pattern is consistent with expression in the perinuclear and peripheral endoplasmic reticulum, and at the cell surface. Expression in the endoplasmic reticulum is expected in an overexpression system. Under high power microscopy, expression at the plasma membrane is seen as a smooth ring-like pattern near the cell surface. Careful comparison of the phase-contrast and fluorescence images also shows undetectable protein localizing to the vacuole. Notably, all recombinant proteins localized to the same subcellular compartments in yeast cells. Hence, functional differences in yeast cells transformed with different constructs were not due to differences in subcellular localization.

To investigate whether Slc11a1, Slc11a2-nonIRE, or Slc11a2-IRE could complement the EGTA-sensitive phenotype of the smf1Δ/smff2Δ/smff3Δ yeast strain, positive Slc11a1, Slc11a2-nonIRE, or Slc11a2-IRE transformants were plated onto YNB plates with different concentrations of EGTA (Fig. 1E). Slc11a2-nonIRE protein expression complemented the EGTA-induced growth deficiency (Fig. 1E, dotted triangle) as previously reported (19, 20, 38). The growth deficiency of smf1Δ/smff2Δ/smff3Δ was also complemented by expression of the Slc11a2-IRE protein, which has not previously been expressed in yeast (Fig. 1E, dashed triangle). No complementation of the EGTA-induced growth deficiency was observed in cells expressing Slc11a1-HA (Fig. 1E, full triangle). On the contrary, Slc11a1 transformants showed a higher degree of sensitivity to EGTA when compared with smf1Δ/smff2Δ/smff3Δ transformed with empty vector (Fig. 1E, strain 2). This indicates a difference in the function of the Slc11a1 versus the Slc11a2-nonIRE and Slc11a2-IRE proteins, because the three recombinant proteins were expressed at the same level and showed the same localization pattern analyzed by immunofluorescence. Because Slc11a2-nonIRE and Slc11a2-IRE were essentially the same in the EGTA sensitivity assay, only Slc11a2-nonIRE was taken forward in further investigations of transport function.
Slc11a1 but Not Slc11a2 Complements Divalent Cation Stress Phenotype in the Bsd2/Rer1 Δ Knock-out Yeast Strain—Using electrophysiology and direct metal ion transport assays we previously demonstrated that Slc11a1 functions as an antiporter in frog oocytes (18). Together with the observation here that Slc11a1 does not complement the EGTA sensitivity of the smf1Δ/smffΔ/smffΔ yeast strain, this indicates that the transport function of Slc11a1 is different from that of Slc11a2-nonIRE and Slc11a2-IRE. A heavy metal cation stress assay was therefore developed to determine whether Slc11a1 could rescue a metal-sensitive phenotype by facilitating transport of metals out of the yeast cell. A metal-sensitive phenotype of the single and double bsd2Δ/rer1Δ yeast strains was established on minimal medium with different concentrations of Mn²⁺, Mg²⁺, Ca²⁺, Cu²⁺, Cd²⁺, and Zn²⁺. The most clear phenotype was seen for the double mutant bsd2Δ/rer1Δ on Cd²⁺ or Zn²⁺ (Fig. 2A), and this strain was therefore selected for further studies. The bsd2Δ/rer1Δ strain was transformed with HA-tagged Slc11a1 and Slc11a2-nonIRE and expression examined by Western blotting (Fig. 2B). Specific immunoreactive bands of 45–55 and 55–65 kDa were detected for Slc11a1-HA (lane 1) and for Slc11a2-nonIRE-HA (lane 2), respectively. The subcellular localization of GFP-tagged proteins was examined by confocal microscopy. As shown in Fig. 2C, GFP-tagged Slc11a1 and Slc11a2-nonIRE exhibited a similar localization pattern indicating that the two proteins localize to the same subcellular compartments in yeast cells, as expected to the endoplasmic reticulum around the nucleus and at the periphery, and importantly at the cell surface (Fig. 2C). As before (Fig. 1D), careful comparison of the phase-contrast and fluorescence images shows undetectable protein localizing to the vacuole. The fluorescence intensity was the same for the Slc11a1 and Slc11a2-nonIRE transformants, in agreement with the Western blot results for the positive transformants.

Serial dilutions of positive transformants were seeded onto plates containing various concentrations of Cd²⁺ or Zn²⁺ (Fig. 2D). The HA-tagged Slc11a1 protein clearly complemented the Cd²⁺- and the Zn²⁺-sensitive growth deficiency of the bsd2Δ/rer1Δ mutant (Fig. 2D, white boxes), whereas Slc11a2-nonIRE did not complement the Cd²⁺- or Zn²⁺-induced growth deficiency (Fig. 2D, lane 4). Given our previous frog oocyte data (18), the most plausible explanation for these results in yeast is that the Slc11a1 protein functions as a divergent cation/proton antiporter, transporting divalent metal cations from the cytoplasm of the yeast to the medium against the proton gradient. We therefore predicted that cytosolic Zn²⁺ levels would be

FIGURE 1. Slc11a2 but not Slc11a1 complements EGTA sensitivity of smf knock-out yeast. A, wild type and 7 possible smf knock-out strains: 1) wild type 6210; 2) 6211; 3) smf1Δ; 4) smf2Δ; 5) smf3Δ; 6) smf1Δ/smffΔ; 7) smf1Δ/smffΔ/smffΔ; 8) smf2Δ/smffΔ; and 9) smf1Δ/smffΔ/smffΔ. b, strains (as in A) grown on media containing copper or manganese or transformed with full-length SMF2. C, immunoblotting with anti-HA of the C-terminal HA-tagged Slc11a1, Slc11a2-nonIRE, and Slc11a2-IRE fusion proteins expressed in smf1Δ/smffΔ/smffΔ yeast. Positions of reference molecular mass markers are indicated in kDa. D, representative fields of fluorescence and corresponding interference contrast images for GFP-tagged Slc11a proteins expressed in yeast. E, streaks of smf1Δ/smffΔ/smffΔ transformants on plates containing 0 or 18 mM EGTA: 1) wild type 6210 empty vector; 2) smf1Δ/smffΔ/smffΔ empty vector; 3) Slc11a1-transformed; 4) Slc11a2-nonIRE-transformed; 5) Slc11a2-IRE-transformed; 6) full-length HA-tagged SMF2 as a positive control. Plates were incubated 3 days at 30 °C. Results are representative of 3 independent experiments.
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lower in Slc11a1 expressing cells compared with non-transformed cells and cells expressing Slc11a2. A method to indicate levels of intracellular labile Zn\(^{2+}\) by measuring \(\beta\)-galactosidase activity was implemented, using a ZRE-lacZ reporter construct, previously developed to monitor Zn\(^{2+}\) levels in yeast cells (39). The Zap1 transcription factor is activated in zinc-limited cells, whereas it is repressed under zinc-replete conditions. When activated, Zap1 binds to zinc responsive elements (ZREs) in its target promoters (40, 41), initiating transcription of the Zap1-regulated genes. The ZRE-lacZ reporter construct was expressed in wild type cells transformed with Slc11a1, Slc11a2, nonIRE, or empty HA-vector. Results show that there was a significantly (\(p < 0.05\)) higher \(\beta\)-galactosidase activity in cells expressing Slc11a1 when compared with cells expressing Slc11a2 or empty HA-vector (Fig. 2E). This indicates that there is a higher rate of ZRE-lacZ transcription in cells expressing Slc11a1 as a result of higher levels of activated Zap1 transcription factor, reflecting low intracellular Zn\(^{2+}\) concentrations. As a control for the Zn\(^{2+}\) specificity of the ZRE-lacZ transcription levels, a HIS4-lacZ control reporter construct was used and no significant differences in the \(\beta\)-galactosidase activity between each group of transformants were observed (data not shown).

Divergence of Slc11a1-like Sequence in Slc11a Homologues—Having developed a divalent cation stress assay that could positively distinguish Slc11a1 from Slc11a2 functions in yeast, it was of interest to determine when this functional difference, and possible sequence changes mediating the change in function, evolved. Previous studies suggested that Slc11a1 and Slc11a2 are paralogues that originated through a gene duplication event in early vertebrate evolution (42, 43). Comparisons of the full-length, N- and C-terminal deduced amino acid sequences of Slc11a homologues from the various organisms were computed on the basis of pairwise alignments (matrixes are available in the supplemental materials). This showed that the core region containing 12 TMDs and the transport motif is the most conserved domain, whereas the level of similarity is markedly lower in both the N and C termini of the proteins. The hypothesized evolutionary relationship of the various Slc11a homologues based on full-length amino acid sequence comparison is shown in the maximum likelihood tree (Fig. 3). Interestingly, the fish homologues all cluster in a monophyletic group and form a clade with the avian and mammalian Slc11a2 homologues together with one of the amphibian Slc11a homologues from Xenopus tropicalis. The newly identified X. laevis Slc11a1, and the X. tropicalis Slc11a1 located to scaffold 77 in the X. tropicalis genome data base, cluster together with the avian and mammalian Slc11a1 homologues. These newly identified amphibian homologues are therefore referred to as Slc11a1 in the phylogenetic tree.
**FIGURE 3. Phylogenetic tree of Slc11a family.** Tree constructed using full-length amino acid sequences. Black boxes indicate Slc11a homologues identified in this study. Bold indicates previously identified Slc11a homologues for which functional data were obtained in this study. Quartet sampling and Neighbor-joining parameter estimation was carried out using TREE-PUZZLE, version 5.0. Maximum likelihood analysis was performed with 1000 puzzling steps. Quartet puzzling support values indicated at the branch nodes. The outgroup was *Mycobacterium tuberculosis* Mramp (37).

Evolution of Functional Ability to Rescue the Divalent Cation Stress Phenotype Post-dates Sequence Divergence—Slc11a homologues were cloned from various organisms and expressed in the smf1Δ/smf2Δ/smf3Δ and the bsΔ2/rer1Δ yeast strains to determine whether antiport function is an ancestral or a derived character: the Slc11a homologue from fruit fly, *malvolio* (44), Slc11a-a and Slc11a-b from trout (*O. mykiss*) (45), Slc11a-a from pufferfish (*F. rubripes*) (42), the Slc11a1-like gene from *X. laevis*, SLC11A1 (46–49) and SLC11A2 from chicken, and human SLC11A1 (50–52) and SLC11A2 (51, 52). Full-length chicken SLC11A2 had not previously been cloned but is known to play a role in the dietary iron uptake in birds (53). All Slc11a homologues were expressed at equal levels in the two yeast strains and localized to the same subcellular compartments (examples for some homologues are shown in Fig. 4, A and B), as previously seen for the murine Slc11a1 and Slc11a2 proteins. Serial dilutions of the various transfectants were seeded onto plates containing either EGTA (Fig. 4C) or cadmium (Fig. 4D). The fruit fly Mvl, fish Slc11a-a and -b, and the *X. laevis* Slc11a1-like proteins all complemented the growth deficient phenotype of the smf1Δ/smf2Δ/smf3Δ strain on EGTA (Fig. 4C, full boxes), arguing that these proteins are Slc11a2-like in function, acting as divalent metal cation symporters. Only chicken SLC11A1 and human SLC11A1 complemented the cadmium-sensitive growth deficiency of the bsΔ2/rer1Δ strain (Fig. 4D, dotted boxes), arguing that these two SLC11A1 proteins function as divalent metal cation antiporters.

**DISCUSSION**

Here we establish that expression of mammalian and avian Slc11a1 homologues, but not mammalian Slc11a2 homologues,
FIGURE 4. Ability of Slc11a1-like homologues to rescue a divalent cation stress phenotype in bsd2 Δ rer1 Δ yeast post-dates Xenopus but pre-dates G. gallus.

A, immunoblotting with anti-HA of some Slc11a homologues expressed in smf1Δ/smft2Δ/smft3Δ yeast. Shown are D. melanogaster Malvolio, X. laevis Slc11a1, G. gallus SLC11A1, G. gallus SLC11A2, H. sapiens SLC11A1, and H. sapiens SLC11A2. Similar results were obtained for the remaining homologues, and for all homologues expressed in bsd2 Δ rer1 Δ yeast (data not shown). Positions of reference molecular mass markers are indicated in kDa.

B, representative fields of fluorescence and corresponding interference contrast images for GFP-tagged Slc11a homologues D. melanogaster Malvolio, O. mykiss Slc11a1-b, and X. laevis Slc11a1 expressed in smf1Δ/smft2Δ/smft3Δ yeast and H. sapiens SLC11A2 expressed in bsd2 Δ rer1 Δ yeast. C, serial dilutions of smf1Δ/smft2Δ/smft3Δ transformants grown on plates with no EGTA or 18 mM EGTA. On each plate, wild type yeast, murine Slc11a1, and Slc11a2-nonIRE were included as internal plate controls, along with D. melanogaster Malvolio, O. mykiss Slc11a1-a and Slc11a1-b, T rupripes Slc11a1-a, X. laevis Slc11a1-like protein, G. gallus SLC11A1/2, and human SLC11A1/2 as shown. Lane numbers refer to specific controls or transformants as indicated below each image. D, same as in C with serial dilutions of bsd2 Δ rer1 Δ transformants grown on plates containing no metal or 6 µM cadmium. Plates were incubated at 30 °C for 4 days. Data are representative of three to four independent experiments.
can rescue *bsd2Δ/rer1Δ* yeast from heavy metal ion stress. Indirect evidence that divalent cations were being transported out of the yeast by Slc11a1 under heavy metal stress was provided by demonstration, using the ZRE-*lacZ* reporter construct, that Slc11a1 expressing yeast cells had lower intracellular Zn\(^{2+}\) than non-transformed cells and cells expressing Slc11a2. These results are analogous to our demonstration (18) that murine Slc11a1 and human SLC11A1 expressed in frog oocytes flux divalent cations in either direction across the oocyte membrane against a proton gradient. At raised extracellular pH (i.e., low proton concentration) there was a net influx of divalent cations into frog oocytes; at low extracellular pH (i.e., high proton concentration) there was a net efflux of divalent cations from frog oocytes. Furthermore, for pre-acidified frog oocytes, a measurable current was induced by addition of divalent cations extracellularly, concomitant with a rise in intracellular pH. These observations led us to conclude that murine and human Slc11a1/SLC11A1 molecules behaved as proton/divalent ion antiporters. Given these prior observations in frog oocytes, and the demonstration here using the ZRE-*lacZ* reporter construct that Slc11a1 expressing yeast cells had lower intracellular Zn\(^{2+}\) than non-transformed cells and cells expressing Slc11a2, the most plausible interpretation of our yeast data is that Slc11a1 is acting as an antiporter to transport divalent metal cations out of the yeast cell under heavy metal stress conditions.

**FIGURE 5.** The N-terminal and protein core of Slc11a proteins determine transport function. A, amino acid alignment of the murine Slc11a1 and Slc11a2 non-IRE with black bars showing the borders defining the N terminus, the core, and the C terminus. The dashed bar (see arrow) shows the border defining the C-terminal region of (20). The gray boxes indicate putative functional differences between the Slc11a1 and Slc11a2 sequence (see text). A full alignment of all Slc11a homologues is available online (see supplemental materials). The alignment was created using ClustalW; * indicates identical amino acids, double dots conserved substitutions, and a single dot semi-conserved substitutions. B, schematic representation of the 6 different Slc11a1/Slc11a2 chimaeras with Slc11a1 depicted in white and Slc11a2 depicted in black. C, serial dilutions of *smf1Δ/smf2Δ/smf3Δ* transformants grown on plates containing no EGTA or 18 mM EGTA. On each plate, wild type yeast, murine Slc11a1, or Slc11a2-nonIRE were included as internal plate controls as indicated. White boxes indicate complementation. D, same as in C with serial dilutions of *bsd2Δ/rer1Δ* transformants as indicated.
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therefore support the hypothesis that mammalian and avian Slc11a1 homologues act as antiporters to transport divalent cations against a proton gradient. In contrast, avian and mammalian Slc11a2 subfamily members, but not mammalian or avian Slc11a1, were able to rescue the EGTA sensitivity phenotype in smn1Δ/smnl2Δ/smnl3Δ yeast. This was likewise consistent with earlier studies of mammalian Slc11a2 subfamily members in yeast (19, 20) and frog oocytes (16) that concluded that mammalian Slc11a2 homologues act as symporters to transport divalent cations along a proton gradient. This functional difference explains the previous findings of murine Slc11a1 and human SLC11A1 to complement EGTA or alkaline pH-sensitive phenotypes in S. cerevisiae (19) or Schizosaccharomyces pombe yeast mutants (20), respectively.

Results presented here lie at the core of controversy about the mechanisms of action of mammalian Slc11a1 versus Slc11a2 subfamily proteins (6, 18, 22–24, 54–56). The data presented here for yeast are clear: mammalian and avian Slc11a1 subfamily members rescue heavy metal ion stress in bsd2Δ/ rer1Δ yeast, whereas Slc11a2 subfamily members cannot. Conversely, Slc11a2 subfamily members rescue EGTA sensitivity in smn1Δ/smnl2Δ/smnl3Δ yeast, whereas Slc11a1 subfamily members cannot. Furthermore, there is clear association between the ability to rescue the metal ion stress phenotype in bsd2Δ/ rer1Δ knock-out yeast and the evolution of avian and mammalian Slc11a1 subfamily members. Our studies are the only ones to demonstrate a divalent metal cation transport phenotype in yeast (this study) or frog oocyte (18) expression systems. What factors may account for differences in interpretation of the function of Slc11a1 studied in mammalian cells compared with the yeast and frog oocyte experiments?

First, most studies in mammalian cells are complicated by vesicular localization of the proteins. Kuhn et al. (23) got around this by isolating phagosomes from macrophages and measuring uptake of iron across the isolated phagosome membrane. They compared phagosomes isolated from macrophages stably expressing wild type Slc11a1 Gly^{469} versus mutant/ functionally null Slc11a1 Asp^{469}, and observed greater import of iron into phagosomes from wild type macrophages. They also found that phagosomes isolated from wild type cells pre-labeled with {sup}55Fe-citrate before phagocytosis contained up to four times more iron compared with phagosomes from mutant cells, and that treatment of wild type macrophages with the lysomotropic agents chloroquine or ammonium chloride reduced the import of iron significantly (23, 24). Multiple groups have compared intracellular iron levels and determined that mutant macrophages have higher cytoplasmic iron than wild type macrophages (6, 54, 55, 57, 58). Macrophages expressing wild type Slc11a1 also have a greater capacity to recycle iron (6), in particular when iron is acquired by Fe receptor-mediated phagocytic uptake of transferrin/anti-transferrin complexes that are delivered to Slc11a1-positive late endosomes and lysosomes, and not when iron is delivered to early endosomes by transferrin-receptor-mediated uptake. Iron acquired by phagocytosis and degradation is retained within the phagosomal compartment in wild type macrophages, and iron release triggered by nitric oxide is by direct secretion of phagosomal contents rather than via the cytoplasm (6). Studies in primary neutrophils also demonstrate that SLC11A1 is expressed in tertiary granules that are positive for the matrix enzyme gelatinase and the membrane subunit of the vacuolar H^{+}-ATPase and can be recruited for exocytosis by treatment of neutrophils with phorbol myristate acetate (5). Higher cytoplasmic iron in mutant macrophages is consistent with a failure of Slc11a1 to recycle iron into late endosomes/lysosomes for exocytosis, and leads to down-regulation of expression of multiple genes through mRNA instability, including Slc11a1 itself (57, 58).

At variance with this series of studies in mammalian macrophages are the data of Jabado et al. (22) who measured intracellular Mn^{2+} flux across phagosomal membranes of intact macrophages. A divalent cation-sensitive fluorescent probe was designed and covalently attached to zymosan. Quenching of phagocytosed zymosan-FF6 probe by extracellular addition of 100–500 μM Mn^{2+} was used to monitor the flux of divalent cations across the phagosomal membrane. Phagosomes from wild type mice extruded Mn^{2+} faster than phagosomes from mutant mice, a difference that was eliminated when acidification of the phagosomal lumen was dissipated. These authors concluded that divalent metal transport through Slc11a1 was H^{+} dependent, and that the direction of metal ion flux under the experimental conditions employed was out of the phagosome into the cytoplasm. By insertion of an HA tag into the extracellular loop between TMD7 and TMD8 Forbes and Gros (56) found that Slc11a1 was mis-targeted to the plasma membrane of Chinese hamster ovary cells, allowing direct study of transport function at the plasma membrane. Using either the metal-sensitive fluorophors calcein and Fura2, or {sup}55Fe^{2+} and {sup}54Mn^{2+} radioisotopes, these authors demonstrated uptake of Fe^{2+}, Mn^{2+}, and Co^{2+} into HA-tagged Slc11a1-transfected cells. Slc11a1 transport was dependent on time, temperature, and acidic pH, with metal ion transport occurring down the proton gradient. Forbes and Gros (56) also concluded that Slc11a1 was a proton/divalent cation symporter, transporting divalent cations down a proton gradient.

Importantly, none of the studies undertaken in mammalian cells to measure Slc11a1-mediated divalent cation transport has looked at the influence of experimental conditions on the expression of endogenous Slc11a2 isoforms, or other molecules such as hepcidin (59, 60) and ferroportin 1 (61, 62) that are known to be involved in regulating iron traffic at the phagosome and plasma membranes in macrophages. In studies looking at iron transport, in particular, changes in intracellular iron will feed back on expression of Slc11a2-IRE isoforms, hepcidin and ferroportin 1. Interestingly, ferroportin 1 is induced by copper (63), another divalent cation transported by Slc11a1 family members (16). Another interesting facet of the antiport activity that we propose is that divalent cations can be fluxed in either direction across the membrane, dependent upon proton and divalent cation concentrations on either side of the membrane. This could account for the observation by Zwilling et al. (64) that addition of small, but not large, quantities of iron to resident macrophages from Slc11a1 wild type mice stimulated catalase- and mannitol-inhibitable antimicrobial activity within a very narrow dose range, leading these researchers to propose that Fe^{2+}-mediated stimulation of the Fenton/Haber-Weiss reaction contributed to antimicrobial activity through the gen-
vation of hydroxyl radicals. Although subsequent studies (65) indicate that this mechanism might not contribute to Slc11a1-controlled antimicrobial activity in vivo, at least in relation to the role of gp91^phox in generating the superoxide anion required for the generation of oxygen radicals, the generation of hydroxyl radicals has been shown by others (57, 66, 67) to contribute to increased protein kinase C and mitogen-activated protein kinase activities in Slc11a1 wild type macrophages that might contribute to pleiotropic effects by increasing transcription and mRNA stability.

Having developed a system in yeast that could be used to measure functional differences in transport activity of Slc11a family members we studied a range of Slc11a orthologues and showed that symport activity is ancestral, and that Slc11a1-like sequence diversity preceded the evolution of antiport activity. A key question is whether antiport activity co-evolved in the vertebrate line to support life on land, or whether it evolved in birds and mammals specifically as a mechanism for resistance again intracellular pathogens? The influence of Slc11a1 on efficiency of iron recycling by macrophages (6, 55) supports the hypothesis that Slc11a1 may have evolved with land-dwelling vertebrates to facilitate iron recycling from effete red cells. Hence, the influence of Slc11a1 on resistance to intracellular infection may be a by-product of, rather than the driving force for, evolution of the Slc11a1 subfamily. Functional analysis of a reptilian Slc11a homologue with Slc11a1-like sequence, which was not available for this study, would be interesting to address this question.

Data obtained with chimeric proteins demonstrated that the N terminus and the core of both of the murine Slc11a1 and the Slc11a2 proteins are independently important for transport activity. This suggests that there might be some pivotal interaction between the N terminus and the core of Slc11a proteins, as such occurs in the CPX-type copper-transporting P-type ATPases (68, 69). Although there are no obvious ion-encoding amino acids in the N terminus of Slc11a proteins, it was recently shown that deletion of the tyrosine-based Slc11a2-specific motif YSCI-62 to 65 inactivates transport function in mammalian cells but does not affect the subcellular localization (70). This motif is conserved in all mammalian Slc11a2, but not Slc11a1, homologues and might therefore play a role in determining symport function. It is also possible that the N termini of Slc11a1 or Slc11a2 mediate protein-protein interactions with other proteins that regulate transport function. However, this would require orthologous regulatory proteins that bind specifically to Slc11a1 or Slc11a2 to be endogenously expressed and functional in yeast, making this a less likely explanation for results obtained here. One fortuitous difference between the chimeric SLCLC11A/SLOC11A proteins used by Tabuchi et al. (20) and those used here was the position at which the C-terminal chimeric construct was made. This allowed us to identify key amino acid changes between Slc11a1 and Slc11a2 subfamily members that might influence transport function (see supplementary information). This included amino acid substitutions at positions (relative to the murine Slc11a2 sequence) 393 in the conserved transport motif in loop 8 of the proteins, at position 403, and at positions 507/508. Further mutagenesis and functional studies will be required to determine the influence of these substitutions on function. A complete understanding of the underlying function of Slc11a1-mediated metal transport also requires high-resolution structural data and more detailed functional studies of binding and transport kinetics. The development here of yeast complementation assays that can readily distinguish antiport versus symport functions in Slc11a family members should facilitate further analysis of transport function in this important family of divergent cation transporters.

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