Nramp2 (DCT1/DMT1) Expressed at the Plasma Membrane Transports Iron and Other Divalent Cations into a Calcein-accessible Cytoplasmic Pool*

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Nramp2, also known as DMT1 and DCT1, is a 12-transmembrane (TM) domain protein responsible for dietary iron uptake in the duodenum and iron acquisition from transferrin in peripheral tissues. Nramp2/DMT1 produces by alternative splicing two isoforms differing at their C terminus (isoforms I and II). The subcellular localization, mechanism of action, and destination of divalent cations transported by the two Nramp2 isoforms are not completely understood. Stable CHO transfecteds expressing Nramp2 isoform II modified by addition of a hemagglutinin epitope in the loop defined by the TM7–TM8 interval were generated. Immunofluorescence with permeabilized and intact cells established that Nramp2 isoform II is expressed at the plasma membrane and demonstrated the predicted extracytoplasmic location of the TM7–TM8 loop. Using the fluorescent, metal-sensitive dye calcein, and a combination of membrane-permeant and -impermeant iron chelators, Nramp2 transport was measured and quantitated with respect to kinetic parameters and at steady state. Iron transport at the plasma membrane was time- and pH-dependent, saturable, and proportional to the amount of Nramp2 expression. Iron uptake by Nramp2 at the plasma membrane was into the nonferritin-bound, calcein-accessible so-called "labile iron pool." Ion selectivity experiments show that Nramp2 isoform II can also transport Co²⁺ and Cd²⁺ but not Mg²⁺ into the calcein-accessible pool. Parallel experiments with transfectants expressing the lysosomal Nramp1 homolog do not show any divalent cation transport activity, establishing major functional differences between Nramp1 and Nramp2. Monitoring the effect of Nramp2 on the calcein-sensitive labile iron pool allows a simple, rapid, and nonisotopic approach to the functional study of this protein.

The Nramp2 gene (natural resistance-associated macrophage protein-2), also known as DCT1 (1) and DMT1 (2), was first identified in mammals (3) and belongs to a large family of integral membrane proteins highly conserved throughout evolution, from bacteria to man (4–8). Structural similarity in the Nramp family translates into functional homology because several members have been shown to function as divalent metal transporters (4, 8–11). Computer-assisted sequence analysis of Nramp2 protein predicts a polytopic membrane protein composed of 12 transmembrane (TM) segments, a glycosylated extracytoplasmic loop, a consensus transport signature (found in several prokaryotic and eukaryotic transport proteins), and precisely conserved charged amino acids in TM domains (4). However, the membrane topology of Nramp2 has yet to be experimentally verified. The Nramp2 gene produces by alternative splicing of the 3'-terminal exon two distinct mRNAs that are distinguished by different C-terminal amino acid sequences and by the presence (isoform I) or absence (isoform II) of an iron response element (IRE) located in the 3'-untranslated region of the mRNA (12). Nramp2 isoform I protein (13) is expressed at the duodenum brush border where its expression is regulated by dietary iron (14). Mutations (G185R) at Nramp2 in mice (nk) and rats (b) cause a severe form of iron deficiency and microcytic anemia, associated with impaired iron absorption at the intestinal mucosa (15–19); together, these results have indicated that Nramp2 isoform I is responsible for iron transport from the duodenum lumen into the cytoplasm of epithelial cells. However, plasma membrane staining for Nramp2 has been difficult to ascertain (20, 21) except at the intestinal brush border of iron-depleted animals (14). Subcellular localization studies of endogenous protein in Hep-2 cells, as well as studies using stably transfected CHO and RAW cells, show that Nramp2 is also expressed in a subcellular vesicular compartment identified as early (20, 21) or late endosomes (22) or both. These findings have suggested that Nramp2, and more specifically its isoform II, may also be implicated in iron acquisition in peripheral tissues as well, transporting transferrin-bound iron across the membrane of acidified endosomes into the cytoplasm (20, 21). This possible role for Nramp2 isoform II in iron transport at the plasma membrane or in acidified endosomes has yet to be explored.

The mechanistic basis of transport has been analyzed in Xenopus oocytes where Nramp2 (DCT1) isoform I transports a number of divalent cations such as Fe²⁺, Zn²⁺, Cd²⁺, Co²⁺, and Cu²⁺. This transport is pH-dependent, electrogenic, and associated with the symport of a single proton (1). Nramp2-mediated iron transport was also demonstrated at the apical membrane of Caco-2 cells (23). In addition, transient overexpression of the wild type but not G185R Nramp2 in HEK293T cells

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† The abbreviations used are: TM, transmembrane; IRE, iron response element; CHO, Chinese hamster ovary; LIP, labile iron pool; FAS, ferrous ammonium sulfate; SHI, salicylaldehyde isocitoxyl hydrazonye; HES-DFO, 6-desferrioxamine; PCR, polymerase chain reaction; HA, hemagglutinin; PBS, phosphate-buffered saline; BSA, bovine serum albumin; AM, acetoxymethylester; MES, 4-morpholineethanesulfonic acid; PM, plasma membrane.

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results in a robust stimulation of cellular $^{55}\text{Fe}^{2+}$ uptake (21). The cellular compartment to which iron is delivered by Nramp2 has not yet been established. Indeed, transport studies using $^{55}\text{Fe}$ as a ligand monitor total cellular accumulation and do not distinguish between free iron, ferritin-bound iron, and iron sequestered in subcellular organelles. Although electrophysiological measurements in Xenopus oocytes are extremely useful to elucidate the bioenergetics and mechanism of transport, such experiments do not recreate the normal environment and subcellular compartments of mammalian cells.

To gain further insight into the structure and function of Nramp2 (isoform II), including the site of transport and destination of the transported substrate, CHO cell clones that stably express an epitope tagged copy of Nramp2 (isoform II) were created. Nramp2 (isoform II) was expressed at the plasma membrane and the epitope tag inserted between TM7 and TM8 was found to be accessible from the medium, indicating that the corresponding loop is indeed extracellular. Transport studies using the metal-sensitive and fluorescence dye calcein demonstrate that Nramp2 (isoform II) can indeed function as a pH-dependent divalent cation transporter at the plasma membrane acting on Fe$^{2+}$, Co$^{2+}$, and Cd$^{2+}$. Our results also show that the incoming iron transported by Nramp2 (isoform II) is delivered to the cytoplasm of CHO cells, within the so-called “labile iron pool” (LIP) (24).

**EXPERIMENTAL PROCEDURES**

**Materials**—Calcein acetoxyethyl ester (calcein-AM) (500 µM stock solution in Me$_2$SO) was obtained from Molecular Probes (Eugene, OR). Ionophore A-23187 (1 mM stock solution in Me$_2$SO) was from Sigma-Aldrich. Stock Fe$^{2+}$ aqueous solutions (20 mM) were always prepared fresh as ferrous ammonium sulfate (FAS; Sigma), NiCl$_2$, CoCl$_2$, CdCl$_2$ (Sigma), MnCl$_2$ (BDH laboratory, Poole, UK), and MgCl$_2$ (Fisher) were prepared as 20 mM stock solutions in water. The membrane-permeant iron chelator salicyladehyde isocotinoyl hydrazonye (SIH; 25 mM stock) was prepared in water and stored at 4 °C. The iron chelator HES-DFO (6 desferrioxamineM, 50,000 starch molecule; 38 mM stock) was prepared in water and stored at −20 °C. SIH and HES-DFO were a generous gift of Dr P. Ponka (McGill University, Lady Davis Institute, Montreal, Canada). $^{55}$FeCl$_3$ (38.49 mCi/mg) was purchased from PerkinElmer Life Sciences.

**Plasmids**—The full-length cDNAs for murine Nramp 1 (GenBank accession number L13732) and Nramp 2 (GenBank accession number L33415) cloned in the EcoRI site of pBluescript KS (pN1KS and pN2KS), as well as Nramp1 and Nramp2 cDNAs modified by the in-frame addition of c-Myc tags at their C terminus are described elsewhere (20, 25).

To optimize protein expression in transfected cells, a full-length Nramp2 cDNA (non-IRE isoform of the mRNA) was modified at its 5′ end by removing untranslated nucleotides and introducing a favorable GCCACC Kozak sequence upstream of the initiator ATG codon (26). This was carried out by PCR amplification using oligonucleotides N2EVR (5′-CTTACCCACCATGGTGATTAC-3′) containing the EcorR restriction site from pBluescriptKS polylinker; nucleotides 61–75 from Nramp2 cDNA) and N2Bn1 (5′-GAATTCCAGCATGATGATTAC-3′; nucleotides 861–888 of Nramp2) (bold type indicates Kozak box, and underlined type indicates restriction sites) and plasmid pN2KS as DNA template. The PCR product was digested with EcoRV and BsmI and inserted in the corresponding sites of pN2KS, yielding construct pN2Vpr–Δ5 UTR–KS. Subsequently, a KpnI–SpeI (polylinker restriction sites) fragment from pN2Vpr–Δ5 UTR–KS was cloned in the mammalian expression vector pcB6 (27) modified by elimination of the Sactl and BgIII sites of the polylinker (pN2B6). The pcB6 vector contains a genetic resistance marker (neo) and uses promoter/enhancer sequences of cytomegavirus to direct high levels of expression of cloned cDNAs. We then wanted to insert a hemagglutinin (HA) epitope tag (YPYDVPDYAS; Ref. 28) in frame with the pcB6-derived cDNA, in the N-terminally delineated by the TM7–TM8 interval. This putative loop is poorly conserved among Nramp proteins, suggesting that it may not play a major functional role, and therefore epitope insertion at that site would not result in loss of function. To insert the HA tag, the Nramp2 cDNA was modified by introduction of a BglII site at position 1085 by PCR-mediated mutagenesis, using oligonucleotides N2B20ns (5′-AACAG-CAGCCCCCATGCGATCCTTTTCCAGTGAC-3′; nucleotides 1069–1103) with N2Sacrev (5′-ATGGTGAGCTCTGAGCCGACG-3′; nucleotides 1202–1223) and N2B2gr (5′-GTCACTGGGAAAGAGATCGCATGGGGGCTGCTGTT-3′; nucleotides 1069–1103) with N2XbaI and N2Sacrev and pN2KS as a template. The resulting PCR fragments were gel purified, heat-denatured, annealed, and amplified with N2XbaI and N2Sacrev; The final PCR product was digested with SacI and XhoI (sites underlined) and inserted in the corresponding sites of pN2B6. The construct was digested with BglII, and the HA epitope tag was inserted at that site using a double-stranded oligonucleotide (HAV2ns, 5′-GATCACGATCCTTGAGCCGACG-3′ and HAV2rev, 5′-GATCCGTAGCGTTATCTGCGGCAGTATTGACG-3′) yielding construct pN2HAb6.

**Cell Culture and Transfection**—CHO cells LRT3 (29) were grown in α-minimum essential medium supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 µg/ml streptomycin. All media and medium supplements were purchased from Life Technologies, Inc. CHO cells were transfected with pN2HAb6 by the calcium phosphate coprecipitation method, as described previously (30). Clones of stably transfected were selected in geneticin (G418; 770 µg/ml; Life Technologies, Inc.) for 10–14 days and tested for protein expression by Western blotting and/or by immunofluorescence using the mouse anti-HA tag monoclonal antibody 16B12 (Babco; Berkeley Antibody Company, Richmond, CA).

**Immunoblotting**—Crude membrane fractions from the various cells were prepared as described previously (31). Protein concentration of the membrane fractions was determined by the Bradford assay (Bio-Rad). Proteins were separated on 7% SDS-polyacrylamide gels and transferred by electroblotting to nitrocellulose membranes. Similar loading and similar transfer of proteins to the membranes was verified by staining the blots with Ponceau S (Sigma). The blots were blocked in 10 mM Tris–Cl, pH 8, 150 mM NaCl, 0.05% Tween plus 5% nonfat skim milk for 16 h at 4 °C. Primary antibodies were used as follows: affinity purified rabbit polyclonal anti-mouse Nramp2 (1:100) or mouse monoclonal anti-HA epitope tag (1:1000; Babco). Anti-rabbit and anti-mouse secondary antibodies conjugated to horseradish peroxidase were used for the detection of immune complexes on the immunoblot (PerkinElmer Life Sciences).

**Immunofluorescence**—For studies in permeabilized cells, cells were grown on glass coverslips and fixed with 4% paraformaldehyde in PBS for 30 min at 4 °C. Immunofluorescence was performed as described previously (20) except that blocking was done with 2% bovine serum albumin (BSA) and 20% normal goat serum in PBS. Incubation with the mouse anti-HA monoclonal antibody 16B12 (1:100) was for 1 h at 4 °C. This was followed by incubation with anti-mouse IgG secondary antibody conjugated to rhodamine (1:200; Jackson Immunochemicals Laboratories Inc., Mississauga, Canada), and 20% normal goat serum in PBS. For studies in intact cells, CHO cells were first incubated with the monoclonal anti-HA antibody 16B12 (1:100) in α-minimum essential medium containing 5% goat serum, 1% BSA, and 10 mM HEPES, pH 7.5, for 1 h at 4 °C. The cells were then fixed in 4% paraformaldehyde (30 min at 4 °C), and they were blocked with PBS containing 2% BSA and 20% normal goat serum for 2 h at 37 °C. A secondary antibody (rhodamine-conjugated goat anti-mouse IgG) was applied (1:200) in the same buffer. Immunofluorescence was analyzed with a Nikon microscope using the 60× oil immersion objective.

**Calcein Loading of the Cells and Divalent Metal Transport Assay**—In preliminary experiments, accumulation of calcein in CHO cells and Nramp2 transfectants was measured to ensure equivalent loading of the dye in the various cell clones. Calcein-AM is a membrane-permeant, nonfluorescent molecule that becomes fluorescent upon intracellular cleavage to calcein (membrane-impermeant) by cytoplasmic esterases. Thus, the appearance of a fluorescent signal was monitored continuously during incubation of a reaction mixture consisting of 1 × 10$^5$ cells in 500 µl of HEPES-buffered saline (10 mM HEPES, 150 mM NaCl, pH 7.4) supplemented with 0.125 or 0.250 µM calcein-AM. The cells were kept at 37 °C with gentle stirring, and fluorescence was recorded using a Hitachi F-3010 fluorescence spectrophotometer (excitation, 488 nm; emission, 517 nm; excitation and emission bandpass, 5 nm). The fluorescence signal of calcein fluorescence by divalent metals was measured in intact cells as follows. CHO cells and transfectants (1 × 10$^5$ cells/ml) were loaded with 0.250 µM calcein-AM for 5 min at 37 °C in loading medium (α-minimum essential medium, 1 mM BSA, 20 mM HEPES, pH 7.4). The cells were washed of excess calcein twice in loading medium, and 1-M aliquots (1 × 10$^5$ cells) were transferred to light proof Eppendorf tubes and kept at room temperature until used. Just prior to
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RESULTS
Plasma Membrane Expression of Nramp2 in Transfected CHO Cells—To obtain direct topological data on the membrane arrangement of Nramp2 protein, we modified an Nramp2 cDNA (non-IRE containing, isoform II) by addition of a HA epitope in the segment delineated by putative TM7 and TM8. Should Nramp2 be expressed at the PM and should the TM7/8 loop be extracellular, the HA tag should be recognizable in intact cells by an antibody added to the extracellular milieu. LR73 CHO cells (29) were transfected with an Nramp2-HA expression construct, followed by isolation of Nramp2-HA expressing clones (clones 310, and 310sd) and immunoblotting of membrane fractions with an anti-HA mouse monoclonal antibody (Fig. 1, top panels). Membrane fractions from CHO cells and from a previously described CHO clone expressing a c-Myc-Nramp2 protein (N2–5; Ref. 20) were used as controls. Nramp2-HA is expressed as a broad immunoreactive band of 75–95 kDa (clones 310, 310sd) that is absent from membranes of either nontransfected CHO cells or of the N2–5 transfectant (Fig. 1, top left panel). The authenticity of Nramp2-HA protein was verified by immunoblotting with a rabbit anti-Nramp2 monoclonal antibody (14, 20) (Fig. 1, top right panel) that recognizes both Nramp2-HA (310, 310sd) and c-Myc-Nramp2 (N2–5) proteins. Thus, Nramp2-HA (isoform II) can be expressed in CHO cells, and insertion of the HA tag does not affect maturation or stability. The subcellular localization, including possible PM expression of the Nramp2-HA protein was analyzed by immunofluorescence in permeabilized and nonpermeabilized cells from clones 310 and 310sd (Fig. 1, A–F). Negative controls included CHO cells (Fig. 1, D–F) and the rhodamine-conjugated secondary anti-mouse antiserum alone (Fig. 1, A–D). In nonpermeabilized Nramp2-HA transfected cells, a ring-like staining at the periphery of the cells was observed with the anti-HA antibody (Fig. 1B), indicating cell surface expression of the epitope (28). In permeabilized Nramp2-HA cells, a similar PM staining for Nramp2 was seen (Fig. 1C), with additional punctate intracellular staining. In both cases, the Nramp2 staining was specific and absent from control CHO cells (Fig. 1, E and F). These results show that (a) Nramp2-HA (isoform II) is expressed at the PM and (b) the HA-tagged

TM7–TM8 loop is extracellular. Expression of Nramp2 (isoform II) at the PM allows functional analysis of its transport properties.

Nramp2 Mediated Transport of Cobalt Monitored Using Calcein—We wished to monitor transport properties of Nramp2 (isoform II) expressed at the PM, using the fluorescent dye calcein. The acetoxymethyl ester of calcein (calcein-AM) is a nonfluorescent, membrane-permeant dye readily taken up by live cells. Once within the cytoplasm, calcein-AM is cleaved by cytoplasmic esterases releasing the membrane-impermeant calcein fluorophor. Calcein fluorescence is stable, insensitive to pH, and can be quenched rapidly and stoichiometrically by divalent metals such as Fe$^{2+}$ and Co$^{2+}$ but not by Cd$^{2+}$ and Mg$^{2+}$ (32). Therefore, calcein should be suitable to measure the activity of a transporter expressed at the PM and transporting divalent metals from the extracellular space to the cytoplasm. Co$^{2+}$ was used initially because it is a strong quencher of calcein fluorescence (32) and a known substrate of Nramp2 in Xenopus oocytes (1). In addition, unlike iron, Co$^{2+}$ valence is stable in aerobic conditions and at different pH (33).

The rate of intracellular calcein accumulation in CHO cells and Nramp2-HA transfectants was first analyzed using two calcein-AM concentrations, 0.125 or 0.25 μM (Fig. 2) (34). Fluorescence appeared at a similar rate in both cell types and was linear over 10 min with total fluorescence emission being proportional to the amount of calcein-AM added, suggesting a nonsaturated process. To verify that calcein is similarly accessible in nontransfected and Nramp2 transfected CHO cells, calcine-loaded Nramp2-HA (Fig. 2B) and control CHO cells

Fe$^{3+}$ Uptake Assay—Iron (final concentration, 1.1 mM; $^{55}$FeCl$_3$; $^{56}$FeSO$_4$ = 1:1.5) was added to ascorbic acid (final concentration, 47 mM) in a molar ratio of 1:44 and kept at room temperature until use. Ascorbic acid was used to promote the formation and maintenance of ferrous (Fe$^{2+}$) iron. Transfected and nontransfected cells were trypsinized, centrifuged, and resuspended in N$_2$-degassed incubation buffer (25 mM Tris, 25 mM MES, 140 mM NaCl, 5.4 mM KCl, 5 mM glucose, 1.8 mM CaCl$_2$, 50 μM ascorbic acid, pH 5.5), at a density of 8 × 10$^6$ cells/ml. An equal volume of incubation buffer containing 20 μM iron (from $^{55}$Fe$^{2+}$/Fe(ascorbic acid mix) was then added to the cell suspension to initiate transport, followed by incubation at either 0 °C (binding) or 20 °C (transport) for 0–30 min. At predetermined time points, aliquots (500 μl) of the cell suspension were transferred to a microcentrifuge tube containing a 200-μl oil cushion (silicon oil:mineral oil = 8:1), and cells were separated from free unincorporated label by centrifugation (20 s at 10,000 g). The walls of the tube were rinsed with cold PBS, and the oil cushion was discarded. The cell pellet was dissolved overnight (20 °C) in 0.1 N NaOH and neutralized by addition of an equal volume of 0.1 N HCl. An aliquot of the cell lysate was counted for radioactivity in a scintillation counter. The protein concentration of individual samples was measured using the Bio-Rad protein assay and used to quantitate $^{55}$Fe incorporation (cpm/μg cell protein).

Measurements, the calcine-loaded cells were centrifuged for 10 s at 10,000 × g and resuspended in 500 μl of transport buffer (150 mM NaCl, 20 mM MES, pH 5–6.5, or 150 mM NaCl, 20 mM HEPES, pH 7–8). The cell suspension was transferred to a stirred thermostated (37 °C) cuvette, and fluorescence measurements were initiated. Divalent metals were added to the cell suspension, and the resulting quench of fluorescence was recorded. Data have been normalized to the steady-state values of fluorescence before addition of the various ligands.
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**Fig. 2. Transport of cobalt in calcein-loaded cells.** A, accumulation of fluorescent calcein in Nramp2 transfected cells (circles) and nontransfected CHO control cells (squares). Briefly, 1 × 10⁷ cells in HEPES-buffered saline, pH 7.4, were incubated with either 0.125 μM (open symbols) or 0.250 μM calcein-AM (filled symbols). The appearance of intracellular fluorescence was monitored continuously in a fluorescence spectrophotometer (excitation wavelength, 488 nm; emission wavelength, 517 nm). Data are the means of three independent experiments. B and C, analysis of calcein/cobalt interaction in permeabilized cells. 2 × 10⁶ calcein-loaded cells/ml in HEPES-buffered saline, pH 7.4, were permeabilized by incubation with ionophore A-23187 (10 μM) and further incubated with increasing concentrations of CoCl₂. Quenching of fluorescence by Co²⁺ was recorded until stabilization of the signal. The results are expressed as the ratio between the fluorescence values recorded at the beginning (before addition of Co²⁺, Fluo init) and at the end of the experiment (Fluo final). Data represent the means ± S.E. of three to five independent experiments for Nramp2 transfected cells and CHO controls (B and C). Linear regression analysis was performed using Sigmaplot 5.0 software. D–F, Nramp2 transport of cobalt at different pH monitored by quenching of calcein fluorescence. Nramp1 and Nramp2 transfected cells were loaded with calcein-AM (0.250 μM) and resuspended in transport buffer adjusted at indicated pH. Co²⁺ (CoCl₂) was added at a final concentration of 20 μM. Results are shown as primary traces for Nramp2 (D) and Nramp1 transfected cells (E) and as histograms (F) representing the means ± S.E. corresponding to the slopes of initial quenching curves of four to six independent experiments.

(Fig. 2C) were permeabilized with the ionophore A-23187 (32) and incubated with increasing concentrations of Co²⁺. After stabilization of the signal, fluorescence was measured and plotted against the Co²⁺ concentration. Results show that calcein fluorescence could be quenched by CoCl₂ in a concentration-dependent fashion (up to 90% could be quenched) and was fairly linear in the range of CoCl₂ concentrations tested. CoCl₂ induced calcein quenching was very similar in CHO (slope after linear regression = −0.164) (Fig. 2C) and Nramp2-HA cells (slope = −0.166) (Fig. 2D), showing that calcein fluorescence is similarly accessible to quenching by divalent cations. To measure the possible transport activity of Nramp2 (isoform II) at the PM, Nramp2-HA transfecteds were loaded with calcein (0.25 μM, 5 min), followed by washing and incubation with Co²⁺ (concentration 20 μM) (Fig. 2D). Nramp2 transport is pH-dependent in Xenopus oocytes (1); thus the effect of Co²⁺ on calcein quenching was measured over time in both cell types and at different pH (5.5 to 8.0). CHO cells expressing the lysosomal Nramp1 homolog (7) were used as negative controls in these experiments (Fig. 2E). The extent of calcein quenching was calculated as the slope of the initial portion (SI) of the graph and representative calcein quenching traces are shown (Fig. 2, D and E). Quenching was also quantified by calculating the slope of the initial portion of the graphs, and averages from four to six independent experiments were calculated (Fig. 2F). At pH 6.5, the Nramp2 expressing clone displays a 11-fold higher rate of Co²⁺-induced quenching of fluorescence when compared with controls, where little if any quenching was noted (SIₐₕ = 0.0077 ± 0.0008 versus SIₐₕ = 0.0007 ± 0.0002; mean ± S.E.). Maximal Co²⁺ uptake by Nramp2 was confined to acidic pH (5.5–6.5) and was completely abolished at pH 8. These results indicate that Nramp2 (isoform II) can transport Co²⁺ at the plasma membrane in a pH-dependent fashion.

**Nramp2 Mediated transport of Fe²⁺ into the Labile Iron Pool**—In these experiments, we wished to (a) determine whether or not Nramp2 (isoform II) can transport Fe²⁺ at the PM and (b) identify the destination of Fe²⁺ transported by Nramp2 at that site. Calcein has been previously used to measure in a dynamic fashion the size of the nonchelated, cytoplasmic LIP (24). Thus, it was of interest to determine whether Fe²⁺ transport by Nramp2 (isoform II) would be into this LIP. For this, CHO cells as well as Nramp1 and Nramp2-HA transfectants were loaded with calcein-AM (0.250 μM, 5 min, 37 °C), followed by washing. Base-line fluorescence was allowed to stabilize (2 min), followed by addition of FAS (20 μM) (Fig. 3A, dotted line 1). FAS had a bi-phasic effect on calcein fluorescence. The first was a very rapid quenching of fluorescence, followed by a slower, time-dependent decrease in fluorescence. These two phases are most obvious in CHO cells and Nramp1 transfectants and correspond to rapid quenching of extracellular and cell associated calcein, followed by slower Fe²⁺ entry into cells and quenching of intracellular calcein. The slope of the initial portion of the curve differed significantly between CHO controls (SIₐₕ = 0.001 ± 0.0006; mean ± S.E.) and Nramp2-HA transfectants (SIₐₕ = 0.0055 ± 0.0005), indicating increased Fe²⁺ entry in the latter cells. After 3 min, the membrane-impermeant iron chelator HES-DFO was added (200 μM; Fig. 3A, dotted line 2). HES-DFO chelates free extracellular Fe²⁺ stopping iron entry into cells but also removes iron from membrane-bound extracellular calcein-Fe²⁺ complexes, causing an increase in cell fluorescence. This increase in cell fluorescence was very similar in wild type and Nramp1 or Nramp2-HA transfected CHO cells and was similar to the rapid quench noted after the addition of FAS to calcein-loaded CHO cells and Nramp1 transfectants (Fig. 3A, dotted line 1). After further stabilization of the fluorescent signal, the membrane-permeant iron chelator SIH was added (250 μM; Fig. 3A, dotted line 3). The ensuing recovery of fluorescence (ΔF;
Fig. 3. **Nramp2-mediated iron transport in transfected CHO cells.** A, untransfected CHO controls (CHO) and Nramp1 and Nramp2 transfected cells were loaded with 0.250 μM calcein-AM. Basal fluorescence was recorded in a spectrofluorometer (excitation, 488 nm; emission, 517 nm). Line 1, addition of 20 μM ferric ammonium chloride; line 2, addition of 200 μM membrane-permeant iron chelator HES-DFO; line 3, addition of 250 μM membrane-permeant iron chelator SIH. ΔF represents the rise in calcein fluorescence corresponding to chelation of intracytoplasmic iron bound to calcein. B, *55Fe* uptake assay. Control CHO cells as well as Nramp1 and Nramp2 transfecteds were incubated at 20 °C in buffer, pH 5.5, containing 10 μM Fe^{2+} (55Fe^2+Fe = 1:15) and 500 μM ascorbic acid. At predetermined times, samples were analyzed for cell-associated radioactivity. Results are expressed as pmol iron/microgram of total cellular protein. C, saturation kinetics of iron transport by Nramp2. Calcein-loaded Nramp2 transfected CHO cells were incubated with 0–10 μM Fe^{2+}. ΔF was calculated and plotted against the iron concentration present in the incubation medium. Data represent the means ± S.E. of three to five independent experiments. D, effect of calcein loading of the cells. E, effect of Nramp2 level of expression on Fe^{2+} transport. D, Nramp2 transfected cells were loaded with 0.125 μM or 0.500 μM calcein-AM, followed by incubation with 1 μM Fe^{2+} (ferrous ammonium sulfate) and membrane-impermeant -and-permeant iron chelator as described in A. E and F, clones expressing various levels of Nramp2 (310–310sd–N2–5; see Fig. 1) together with control CHO cells were analyzed in a similar manner and show the effect of Nramp2 protein level of expression on Fe^{2+} uptake. Results are shown as primary traces (E) and histograms representing mean ± S.E. of ΔF calculated from four independent experiments (F).

Fig. 3A) was representative of the intracellular iron bound to calcein (LIP) and was thus proportional to the amount of iron transported across the plasma membrane. Reproducibly, Nramp2-HA transfecteds exhibited a 3–4-fold higher ΔF than that measured either in CHO cells (ΔF<sub>CHO</sub> = 0.21 ± 0.02 versus ΔF<sub>N2</sub> = 0.68 ± 0.03; mean ± S.E.) or in Nramp1 transfecteds, demonstrating Fe^{2+} transport by Nramp2 (isoform II). These results were confirmed in parallel experiments using *55Fe* as an isotopic ligand (Fig. 3B). Indeed, expression of Nramp2 in CHO cells caused rapid incorporation of *55Fe* leading to a 7-fold increase above background levels seen in CHO and in Nramp1 transfected controls, over a 30-min incubation period.

The characteristics and parameters of Nramp2 (isoform II)-mediated uptake of iron measured by calcein quenching were further examined. First, the effect of increasing concentrations of FAS (final concentration, 0.5–10 μM) in the transport buffer on the amount of intracellular calcein fluorescence quenching (ΔF) was analyzed in Nramp2-HA transfecteds (Fig. 3C). Results show that calcein quenching by iron in Nramp2-HA transfecteds was a saturable process, with saturation occurring at approximately 1 μM (Fig. 3C). At 1 μM FAS, the difference in calcein quenching/iron accumulation between Nramp2-HA transfecteds and CHO controls was maximal at 5-fold (ΔF<sub>CHO</sub> versus ΔF<sub>N2</sub>). To determine whether the amount of calcein loaded in the cells was limiting and/or could affect the modulation of ΔF by Nramp2, two concentrations of calcein were used to load Nramp2-HA transfecteds (Fig. 3D), followed by incubation with 1 μM FAS. Results show that the intracellular calcein concentration affected neither the rate (S<sub>1.000</sub> = 0.0051 versus S<sub>1.500</sub> = 0.0057) nor the extent (ΔF<sub>1.000</sub> = 0.52 versus ΔF<sub>1.500</sub> = 0.51) of quenching of fluorescence by Fe^{2+}, indicating that calcein is not rate-limiting in these experiments. Finally, the effect of different levels of Nramp2-HA protein expression on the measured transport activity was analyzed in CHO transfecteds expressing increasing levels of Nramp2-HA protein (clones N2–5–310sd–310; Fig. 1). Fig. 3E shows a comparison of the primary traces, and Fig. 3F shows a histogram compiling ΔF measurements from four independent experiments. In clones N2–5 and 310sd that express high and comparable amounts of Nramp2-HA, a 5-fold increase in iron up-
take is seen when compared with CHO cells, whereas only a 2.5-fold increase is noted in the lower expressing clone 310. Thus iron transport by Nramp2 (isoform II) at the PM is saturable and is into the cytoplasmic LIP.

Ion Selectivity of Nramp2 Transport at the Plasma Membrane—The specificity of Nramp2 (isoform II) for different divalent metals was investigated next. For this, divalent metals that are not quenchers of calcein fluorescence but may yet act as competitors of iron transport by Nramp2 were used. Thus, possible competition of iron transport was tested in Nramp2 transfectants, using Cd2+ and Mg2+. Cd2+ has previously been described as a substrate for Nramp2 (1) and is an extremely poor quencher of calcein fluorescence (32). Mg2+ was chosen as a negative control because it is not transported by Nramp2 in Xenopus oocytes (1). Cells were loaded with calcein, and either 1 μM (Fig. 4A) or 20 μM (Fig. 4B) iron was added to the cells, together with increasing concentrations of the competing cations, and fluorescence was continuously monitored. After 3 min, cells were sequentially exposed to HES-DFO followed by SIH, and ΔF was determined. ΔF averages calculated from three independent experiments corresponding to loading of the cells with 1 μM Fe2+ and 0.5–10 μM Cd2+ are shown in Fig. 4A. Representative traces obtained by loading the cells with 20 μM Fe2+ and 5–200 μM CdCl2 are shown in Fig. 4B. These results show that iron transport by Nramp2 can be effectively competed by Cd2+. Indeed, ΔF for iron transport is decreased to background levels when a 10-fold molar excess of Cd2+ is present (1 μM iron versus 10 μM Cd2+ and 20 μM iron versus 200 μM Cd2+). Incubating the cells with Mg2+ (100 μM) had no effect on iron transport by Nramp2 (data not shown). Incubating calcein-loaded Nramp2 transfectants with either Cd2+ or Mg2+ in the absence of iron had no effect on the intracelluar calcein fluorescence (data not shown). This confirms that the noted Cd2+-induced inhibition of quenching noted in Fig. 4B is due to competition at the site of transport. Therefore, Nramp2 (isoform II) expressed at the PM is shown to transport, in a pH-dependent fashion, a number of divalent cations directly into a calcein-accessible, cytoplasmic pool.

**DISCUSSION**

One of the distinguishing features of the Nramp protein family is the presence of a 20–46-amino acid residues hydrophilic loop delineated by TM7 and TM8. The sequence of this loop is not conserved throughout evolution; for example mammalian Nramp1 and Nramp2 show 19 of 42 identical residues with nine conservative substitutions in this segment. However, this segment often contains N-linked glycosylation signals (NXS or NXT), suggesting that this loop would be glycosylated and extracytoplasmic (35). This, together with the presence of a transport signature in the adjacent TM9–TM10 interval, which is conserved at the cytoplasmic face of several bacterial periplasmic permeases, was initially used to anchor the predicted topological arrangement of the 12 TM domains of the Nramp protein (4). In this study, the insertion of an epitope tag in the TM8–TM9 loop did not affect protein stability, membrane targeting, or transport properties. In addition, immuno-fluorescence studies in nonpermeabilized Nramp2 CHO transfectants with an anti-tag antibody confirmed that this loop was indeed extracellular in Nramp2. These results provide a first validation of the initial topological model of the protein based on hydropathy profiling and suggest that this approach could be used for topology mapping of individual TM domains of Nramp2.

The mechanism of transport of Nramp2 has so far been studied after expression in Xenopus laevis oocytes (1), in non-transfected or in stably or transiently transfected cultured cells (21, 23) and by the use of radioisotopes. Although those are sound technological approaches there are certain limitations. Studies in Xenopus oocytes require unique expertise and instrumentation and may not fully recreate the transport environment of mammalian cells. 56Fe2+ and 56Fe3+ are high energy emitters that require containment that limit their use. In addition, isotopic iron has a tendency to bind nonspecifically to various cellular components and proteins in live and dead cells, producing a relatively high background in whole cells assays. Also, the rapid oxidation of Fe2+ (substrate) to Fe3+ (nonsubstrate) at various degrees in aqueous solutions complicates analysis of transport assays. Finally, neither method provides information on the destination of the transported iron, because they cannot distinguish between intracellular iron complexed to ferritin, and the cytoplasmic pool of free Fe3+ (LIP). Another limitation of these methods is that the subcellular localization of the protein to a functionally relevant site is difficult to establish with certainty. Calcein binds to a number of divalent cations, Ca2+, Fe2+, Cd2+, Mg2+, and Co2+, and some of these (notably Fe2+ and Co2+) are potent quenchers of calcein fluorescence. In addition, only binding of Fe2+ but not Fe3+ to calcein results in fluorescence quenching (32), alleviating the problems associated with change in valence of the iron atom during transport experiments. Calcein has been previously used to monitor in nontransfected cells the size of the ferritin-free, so-called labile iron pool (24, 36), thus suggesting that it could also be used to monitor the activity of Nramp2 in intact cells, and may also provide information on the status of iron transported by Nramp2. By using a combination of membrane-permeant (HES-DFO) and membrane-permeant (SIH) Fe2+ chelators in calcein-loaded cells, we were able to accurately measure in a kinetic fashion the effect of Nramp2 expression on the size of the intracellular iron pool in transfected CHO cells. We observed robust influx of iron in Nramp2 transfectants that was related to the amount of Nramp2 protein expressed in these clones; over a 3-min loading period, Nramp2-expressing cells showed a 5-fold increase in the initial rate of fluorescence quenching and 3–4-fold increase in total SIH-sensitive fluorescence over control CHO cells (ΔF). In this assay, Nramp2 transport of Fe2+ is pH-dependent (optimum at pH 5.5–6.0) and is saturated at approximately 1 μM, a value in good agreement with that measured in Xenopus oocytes for Nramp2/DCT1-mediated (Km = 2 μM) and SMF1-mediated (Km = 2.2 μM) iron transport (1, 9). Finally, Nramp2 expressed at the plasma membrane is shown to transport Co2+ and Cd2+ but not Mg2+. The fate of iron transported at the plasma membrane by an
iron transporter such as Nramp2 has been debated (reviewed in Ref. 37). It could become quickly complexed to ferritin, sequestered away in subcellular membrane compartments or organelles such as mitochondria or could be part of a free cytoplasmic pool previously identified as the LIP (24). Results from this study show that iron transport into CHO cells by Nramp2 is into the calcein-accessible cytoplasmic LIP. In addition, kinetic and saturation measurements show that most of the iron transported by Nramp2 during the monitoring period is into that pool.

The Nramp2 gene produces by alternative splicing of the 3′-terminal exon two distinct proteins and mRNAs that are distinguished by different C-terminal amino acid sequences and the presence (isoform I) or absence (isoform II) of an IRE located in the 3′-untranslated region of the mRNA (12). The isoform I protein (14) is expressed at the duodenum brush border where it is regulated by dietary iron and ultimately responsible for iron transport from the duodenum lumen into the cytoplasm of epithelial cells. It is the isoform I of Nramp2 that has been used in transport assays in *Xenopus* oocytes to demonstrate the iron transport by Nramp2 (1). Subcellular localization studies in intact cells (Hep-2), in transfected CHO cells, and in RAW mouse macrophages show that Nramp2 is also expressed in a subcellular vesicular compartment identified as early (20, 21) or late endosomes (22). In several cell lines tested (murine erythroleukemia MEL cells and Sertoli TM4 cells), it appears that the majority of the protein expressed at that site is the isoform II of Nramp2. Co-localization studies with transferrin-fluorescein isothiocyanate (20, 21) and in vivo studies in *mk* and *b* mutant animals support a role for Nramp2 in the transport of transferrin iron from acidified endosomes into the cytoplasm of peripheral tissues (38, 39). In the present report, we have been able to establish immunofluorescence that the IRE negative isoform II of the protein (used in our expression construct) can be expressed at the plasma membrane. The strict pH dependence of transport together with the rapid fluorescence quenching kinetics observed in Nramp2 transfectants indicates that Nramp2 isoform II can indeed function as a pH-dependent divalent cation transporter at the plasma membrane of these cells. The fact that both isoforms I and II can be targeted to the PM membranes in primary cells and in transfected cells would suggest that the membrane targeting information required for this process is not located in the extreme C terminus of the protein, which shows no sequence homology between isoforms I and II. Thus, it is interesting to speculate that NPXY and YSFC motifs identified by scrutiny of the N-terminal sequence of Nramp2 may be implicated in this process, because they have been identified as sorting motifs for other membrane proteins such as transferrin receptor, Lamp-1, CD3, and H,K-ATPase (40–42).

The fluorescence quenching method developed here to monitor Nramp2 transport offers several advantages over current methods. First, its does not rely on the use of radiotopisotope derivatives of Nramp2 substrates, some of which are not commercially available. Second, it is carried out in intact mammalian cells and is not destructive, and cells analyzed in this fashion can be further put through other tests. Third, the use of two chelators allows one to easily distinguish nonspecific binding from transport and intracellular accumulation of iron. Fourth, quantitative and rapid kinetic data can be obtained for transport of three divalent cations by this assay. Finally, and most importantly, we show that divalent cations transported by Nramp2, including Fe(II) is into a free cytoplasmic pool that is accessible to calcein. This assay can now be used to identify structure/function relationships in Nramp2 variants generated by site-directed mutagenesis, including identification of residues underlying pH dependence and substrate specificity of the transporter. In addition, insertion of the HA tag in the predicted TM7–TM8 loop does not seem to affect transport activity, compared with Nramp2 marked with a c-Myc tag at the C terminus. Thus, membrane topology of Nramp2 could be determined by immunofluorescence in CHO cells expressing recombinant proteins engineered with epitope tags at different positions and tested for biological activity in this assay (28).

As opposed to their Nramp2 counterparts, CHO cells transfected with and expressing high levels of the macrophage-specific Nramp1 protein (4) do not demonstrate increased divalent cations import in the calcein quenching assay. The high degree of sequence homology between mammalian Nramp1 and Nramp2 (78% sequence similarity), and the report that a loss-of-function mutation at the *mvl* locus can be corrected by Nramp1 in transgenic flies (11) in a manner similar to that of increased dietary metals (38), together strongly suggest that Nramp1 can transport divalent cations as well. The lack of divalent cation transport reported here for Nramp1 CHO cells (Figs. 2E and 3B) is likely to be the result of the absence of Nramp1 expression at the plasma membrane concomitant to strict expression in the lysosomal compartment of these cells (25, 43). The results obtained here with Nramp1 and CHO transfectants clearly suggest that the protein signals underlying targeting of the two proteins to distinct subcellular compartments can be identified in chimeric proteins expressed in CHO cells and analyzed by this assay.

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