Dynamic Traffic through the Recycling Compartment Couples the Metal Transporter Nramp2 (DMT1) with the Transferrin Receptor*

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Nramp2 (natural resistance-associated macrophage protein 2, also called DMT1 and Slc11a2) is a proton-dependent cation transporter, which plays a central role in iron homeostasis. To study the subcellular distribution and dynamics of the transporter, we generated a construct encoding the long splice variant of Nramp2 (isoform II) tagged with the hemagglutinin epitope on a predicted extracellular loop. Cells stably transfected with this construct revealed the presence of Nramp2 in both the plasma membrane and in an endomembrane compartment. By labeling the exofacial epitope with a pH-sensitive fluorescent indicator, we were able to establish that this variant of Nramp2 resides in a vesicular compartment with an acidic lumen (pH 6.2) and that acidification was maintained by vacuolar-type ATPases. Dual labeling experiments identified this compartment as sorting and recycling endosomes. Kinetic studies by surface labeling with ¹²⁵I-labeled antibodies established that the fraction of endomembrane Nramp2 was approximatively equal to that on the cell surface. The two components are in dynamic equilibrium: surface transporters are internalized continuously via a clathrin and dynamin-dependent process, whereas endosomal Nramp2 is recycled to the plasma membrane by a phosphatidylinositol 3-kinase-dependent exocytic process. Depletion of cholesterol had no discernible effect on Nramp2 internalization, suggesting that rafts or caveolae are not essential. Because the pH at the cell surface and in endosomes differs by >1 unit, the rates of transport of Nramp2 at the surface and in endomembrane compartments will differ drastically. Their subcellular colocalization and parallel trafficking suggest that Nramp2 and transferrin receptors are functionally coupled to effect pH-dependent iron uptake across the endosomal membrane.

Iron plays an essential role in several vital processes including the delivery of oxygen to peripheral tissues for utilization in mitochondrial respiration (1). The extracellular and cytosolic concentrations of iron must be regulated stringently to provide appropriate amounts of the metal to a variety of metabolic pathways. Modest deviations can have serious pathological consequences, including common diseases of humans, such as anemia, neurodegenerative diseases, and hemochromatosis, which result from either iron deficiency or overload, respectively (2). Iron homeostasis involves carefully controlled absorption and storage processes, whereas loss of the metal is thought to be an unremitting step. Iron is absorbed at the level of the duodenum after reduction from the ferric to the ferrous state by ferric reductase. Uptake across the duodenal brush border has been attributed to Nramp2, a transmembrane protein originally identified by homology with the natural resistance-associated macrophage protein-1 (Nramp1) (3, 4). The function of Nramp2 as a metal ion transporter was uncovered subsequently, during the course of functional cloning experiments (5). For this reason, Nramp2 was renamed as DMT1 (divalent metal transporter 1) and more recently has been annotated as Slc11a2 (solute carrier family 11, member a2). Iron taken up at the brush border of duodenal cells through Nramp2 is released into the blood stream by IREG1 (6), also called ferroportin1, which translocates the metal across the basolateral membrane. Circulating iron is largely complexed to transferrin, for delivery to peripheral tissues via the transferrin receptor pathway. The critical role of Nramp2 in iron homeostasis is highlighted in two mutants bearing a loss-of-function allele at this locus: the nk mouse and the Belgrade rat carry the same missense mutation (G185R) in Nramp2 (7). Homozygous mk/mk mice present microcytic, hypochromic anemia because of severe deficits in intestinal iron absorption and erythroid iron utilization, presumably as a consequence of impaired ability of Nramp2 to transport iron (8) and/or defective targeting or stability of the mutant protein at the intestinal brush border and in reticulocytes (9–11).

Nramp2 is a glycosylated protein composed of 12 transmembrane domains which has been shown to transport, in a proton-dependent manner, a broad range of divalent metals including Fe⁺⁺, Mn⁺⁺, Co⁺⁺, Cd⁺⁺, Cu⁺⁺, Ni⁺⁺, Pb⁺⁺, and possibly Zn⁺⁺ (5, 12, 13). Two different mRNAs are generated by alternative splicing of a 3’-exon of the Nramp2 gene to produce two distinct proteins with different C-terminal sequences (14). Isoform I (DMT1A) is expressed at the apical membrane of duodenal enterocytes and kidney epithelial cells (5, 15–17). The mRNA encoding isoform I contains an iron-responsive element (IRE) in its 3’-untranslated region, and its abundance is therefore controlled by the prevailing concentration of intracellular iron (13, 18–20). Isoform II (DMT1B) lacks the IRE and differs from isoform I at its C terminus. It is expressed in nonepithelial cells and is particularly abundant in erythroid cells (21).

The subcellular localization of Nramp2 was initially the sub-cellular compartment thought to transport iron, but it has subsequently been demonstrated that Nramp2 is also present at the plasma membrane, particularly at raft domains which has been shown to transport, in a proton-dependent manner, a broad range of divalent metals including Fe⁺⁺, Mn⁺⁺, Co⁺⁺, Cd⁺⁺, Cu⁺⁺, Ni⁺⁺, Pb⁺⁺, and possibly Zn⁺⁺ (5, 12, 13). Two different mRNAs are generated by alternative splicing of a 3’-exon of the Nramp2 gene to produce two distinct proteins with different C-terminal sequences (14). Isoform I (DMT1A) is expressed at the apical membrane of duodenal enterocytes and kidney epithelial cells (5, 15–17). The mRNA encoding isoform I contains an iron-responsive element (IRE) in its 3’-untranslated region, and its abundance is therefore controlled by the prevailing concentration of intracellular iron (13, 18–20). Isoform II (DMT1B) lacks the IRE and differs from isoform I at its C terminus. It is expressed in nonepithelial cells and is particularly abundant in erythroid cells (21).
fect of debate, being variously assigned to either the plasmalemma, early or recycling endosomes, late endosomes, and/or lysosomes (1, 22, 23). Some of this ambiguity has been resolved since the original submission of this manuscript by Tabuchi et al. (24), who realized that the intracellular compartments where isoforms I and II are targeted are not identical: whereas isoform I accumulates in late endosomes/lysosomes, isoform II is found preferentially in early endosomes. In addition, there is general agreement that both isoforms are present at the surface membrane. The relative magnitude of these compartments, however, remains undefined. It is also unclear whether the plasmalemmal and intracellular pools of Nramp2 are constant and static or whether they are in a dynamic equilibrium, in which case alteration of the rates of endo- and exocytosis would result in net changes in the size of the pools. Moreover, the relative contribution of the exocarial and endomembrane transporters to the uptake of metals into the cytosol has not been established. Because transferrin can release iron in acidic environments and because Nramp2 is thought to cotransport the metal with protons (5), most of the transport would be expected to occur in acidic organelles of the endocytic pathway. Yet, the presence of Nramp2 isoform II in an acidic compartment has not been directly documented, nor is it clear whether the isoform I found in presumably acidic late endosomes/lysosomes is functional in this compartment or is en route for degradation.

Although isoforms I and II are expressed preferentially in epithelial and erythroid cells, respectively, they are not mutually exclusive. Indeed, it has recently become apparent that isoform I is also present in blood cells and that isoform II exists in polarized epithelial cells (24–26). It remains to be defined whether the distribution and behavior of the two isoforms differs significantly in epithelial and non-epithelial cells. In the present manuscript we analyze the properties of isoform II of Nramp2. The main objectives were to: (a) compare and confirm the subcellular distribution of isoform II in non-epithelial and epithelial cells, with particular interest in the pH of the intra-cellular compartment and its relationship to the transferrin receptor (TIR); (b) quantify the relative magnitude of the plasmalemmal and endomembrane pools of isoform II; (c) define whether it is stationary or undergoes dynamic intracellular traffic; (d) analyze the mechanism of internalization; and (e) assess its ability to transport metals in the different subcellular compartments. To this end, we generated cell lines expressing isoform II of Nramp2 tagged with an extracellular HA epitope. The accessibility of the exofacial tag enabled us to monitor the traffic of the transporter from the surface membrane to intracellular compartments in live cells. Moreover, by attaching a pH-sensitive fluorophore to the tag, we were able to measure the luminal pH of the intracellular organelles where Nramp2 is present.

**EXPERIMENTAL PROCEDURES**

**Materials**—Monoclonal mouse antibody (HA.11) and its Fab fragment directed against the HA epitope were purchased from BabCo. Mouse anti-Myc antibody was from Santa Cruz. Secondary fluorophore-conjugated antibodies were: fluorescein isothiocyanate-labeled donkey anti-mouse, Cy3-labeled donkey anti-mouse or rabbit antibody, all purchased from Jackson Immunoresearch Laboratories, and Alexa488-coupled anti-mouse antibody from Molecular Probes. Peroxidase-coupled goat anti-mouse antibody was from Pierce. 125I-Radiolabeled sheep anti-mouse antibody was purchased from Amersham Biosciences. The rat anti-Nramp2 monoclonal antibody was obtained from Dr. K. Maren (Department of Biochemistry, University of Georgia, Athens). Rhodamine-labeled transferrin, nigericin, and calcein-AM were also purchased from Molecular Probes. Polyclinac was from Kamiya, and wortmannin was from Calbiochem. CoCl2, tunicamycin, and methyl-β-cyclodextrin were from Sigma. Peptide N-glycanase F and Tauq poly-merase were from New England Biolabs. The first strand cDNA synthesis kit was from Roche Applied Science.

**Cell Culture and Transfection**—Chinese hamster ovary (CHO) cells were grown at 37 °C in a 5% CO2 incubator in α-minimum Eagle’s medium (Invitrogen) and LLC-PK1 cells in Dulbecco’s modified Eagle’s medium–F12 medium, both supplemented with 10% fetal calf serum. For immunofluorescence, immunoprecipitation, or video microscopy, the cells were plated on glass coverslips between 24 and 48 h before the experiment. LLC-PK1, or CHO cells were transfected with Nramp2-ΔHA-pC86 or Nramp2-Myc-pC86 vectors using a calcium-phosphate coprecipitation method (12). Selection of stably transfected clones was done using 1 μg/ml G418 (Invitrogen) for 10 days. Individual colonies were then isolated and expanded. All other vectors used for this study have been described earlier: GFP-KDEL (27), Myc-tagged syntaxin 13 (28), Rab7-GFP (29), clathrin light chain-GFP and dynamin-1 K44A (30). Transfection was performed using FuGENE-6 (Roche Applied Science), according to the manufacturer’s instructions.

**Immunofluorescence**—Cells were fixed with 4% paraformaldehyde for 15 min at room temperature and, where indicated, permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBST). The preparation was blocked for 30 min in medium containing 5% nonfat dry milk, then incubated with the primary antibodies (mouse anti-HA 1:1,000, mouse anti-Myc 1:1,000, rabbit anti-α-mannosidase II 1,500) followed by secondary antibodies (1:1,500) for 1 h each. For experiments in which live cells were exposed to antibodies, a 1:200 dilution was used for both the anti-HA and its Fab fragment, before fixation and staining of the cells. Cells were visualized on a Leica IRE DR2 microscope using a 100× oil immersion objective. Digital images were acquired with an Orca II ER camera (Hamamatsu) driven by the Openlab 3 software (Improvement) installed on an Apple G4 computer. Images were cropped, assembled, and labeled using Adobe Photoshop software. For colocalization studies, 10 optical sections separated by 0.1 μm were deconvolved using the nearest neighbor algorithm of Openlab to remove out-of-focus fluorescence.

**Glycosylation Studies**—After several rinses with cold PBS containing 1 mM each MgCl2 and CaCl2, cells were incubated in lysis buffer (PBS, 1% Triton X-100, plus a protease inhibitor mixture from Sigma) for 30 min at 4 °C. Lysates were subjected to centrifugation at 10,000 × g for 30 min at 4 °C, and 500 units of peptide N-glycanase F was then added to 100 μl of the supernatant. Deglycosylation was allowed to proceed for 1 h at 25 °C, and the resulting sample was analyzed by SDS-PAGE and immunoblotting. An aliquot of untreated lysate was used for comparison. For tunicamycin treatment, 2.5 μg/ml N-glycosylation inhibitor was added directly to the culture medium for 24 h before analysis.

**Immunoblotting**—After separation by SDS-PAGE, proteins were transferred to nitrocellulose, and the membrane was blocked for 1 h in PBST with 5% milk before an overnight incubation at 4 °C with the primary antibody (mouse anti-HA, 1:10,000) in the same solution. After several rinses with PBST, peroxidase-coupled secondary antibody (goat anti-mouse, 1:10,000) was added in PBST with 3% milk for 1 h at room temperature. Immunoreactive proteins were visualized by enhanced chemiluminescence and exposed to Kodak film.

**Quantification Using Radiolabeled Antibody**—For quantification of Nramp2, LLC-PK1 cells stably transfected with HA-tagged Nramp2 (LLC-PK1-Nramp2-HA) were grown to confluence in six-well plates. To determine plasmalemmal Nramp2, the exposed epitopes were saturated by fixing the cells, followed by incubation with anti-HA antibody (1:200) for 1 h at 4 °C. For quantitation of total Nramp2 the cells were first fixed and permeabilized before addition of the primary antibody. Internalization of the protein was followed after saturation of the extracellular epitopes by incubation of intact cells with anti-HA antibody (1:200) for 1 h at 4 °C. After removal of the excess primary antibody, endocytosis of the transporter was allowed to proceed for varying times at 37 °C and then stopped by fixation with 4% paraformaldehyde for 10 min at 37 °C. To quantify recycling of the transporter, the mouse anti-HA antibody was incubated with live cells at 37 °C for different times before fixation and permeabilization. In an attempt to identify the primary antibody bound was quantified after rinsing several-fold, blocking in PBS with 5% sheep serum, followed by incubation for 1 h with 0.5 μg/ml 125I-labeled sheep anti-mouse IgG and additional rinsing. Finally the samples were solubilized in 1 ml of PBS with 1% SDS and the lysate transferred into tubes for γ-counting. The radioactive background was established on parallel samples not exposed to the antibody.

Where specified, cells were serum depleted for 30 min at 37 °C in RPMI (Wisent) and then treated for 1 h with 100 nM wortmannin at 37 °C. Methyl-β-cyclodextrin (10 mM in RPMI) treatment was for 2 h at 37 °C.
Fig. 1. Expression and function of Nramp2-HA. A, endogenous expression of Nramp2 IRE and non-IRE in LLC-PK1 cells. To evaluate the specificity of the reaction, 10 ng of plasmids encoding for either Nramp2 IRE and non-IRE was subjected to PCR using primers designed to amplify exclusively the IRE or non-IRE variants (top panel). In the lower panel, the same set of primers was used to amplify DNA that was reverse transcribed from mRNA purified from untransfected LLC-PK1 cells (three leftmost lanes) or from LLC-PK1, stably expressing Nramp2 non-IRE-HA or Nramp2 IRE-HA (two rightmost lanes in lower panel). Reverse transcriptase or mRNA was omitted where indicated (third and fourth lanes from left, respectively). B, schematic representation of the transmembrane topology of Nramp2, indicating the potential sites of glycosylation as well as the position of the HA epitope in the fourth extracytoplasmic loop. The location of the Myc epitope used in some experiments at the C terminus of the protein is also shown. C, the HA-tagged Nramp2 is glycosylated. LLC-PK1 cells stably transfected with Nramp2-HA were grown in the absence (Control) or presence of 2.5 μg/ml tunicamycin for 24 h. Lysates were obtained from these cells and used for SDS-PAGE and immunoblotting using anti-HA antibodies. Where noted, lysates from control cells were treated with peptide N-glycosidase F (PNGase F) before electrophoresis. Four different species are labeled a–d. Markers indicate molecular mass in kDa. D, Nramp2-HA is functional. The rate of Co2+ uptake was determined flurometrically in untransfected and in Nramp2-HA-transfected LLC-PK1 cells, using calcein, as described under “Experimental Procedures.” Cells were bathed in NaCl medium and, after 1 min 50 μM CoCl2 was added at the indicated pH and recording continued. To facilitate comparison, recordings were normalized to the initial fluorescence, which was very similar in all cases. Traces are representative of four similar experiments.

Cobalt Uptake Measurements—Co2+ uptake was measured by microphotometry, monitoring the metal-induced quenching of the fluorescence of calcein. Measurements were performed using a Nikon Diaphot TMD inverted microscope coupled to the M series dual wavelength illumination and recording system from Photon Technologies, Inc. (South Brunswick, NJ). We used a 490 nm excitation wavelength, a 510 nm dichroic mirror, and a 520 nm emission wavelength. Cells grown on coverslips were incubated in a NaCl-based medium (140 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 1 mM glucose, and 15 mM HEPES, pH 7.3) with 2 μM cell-permeant calcein-AM. Measurements were performed using an Nikon Diaphot TMD inverted microscope coupled to the M series dual wavelength illumination and recording system from Photon Technologies, Inc. (South Brunswick, NJ). We used a 490 nm excitation wavelength, a 510 nm dichroic mirror, and a 520 nm emission wavelength. Cells grown on coverslips were incubated in a NaCl-based medium (140 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 1 mM glucose, and 15 mM HEPES, pH 7.3) with 2 μM cell-permeant calcein-AM. The basal fluorescence level was recorded for at least 1 min before addition of the Co2+-containing transport solution (50 μM CoCl2, 140 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 1 mM glucose, and 15 mM MES, pH 5.5). To facilitate comparison, the fluorescence was normalized in all cases relative to the basal level. Multiple measurements with similar levels of basal fluorescence intensity were performed and used for comparisons. Data presented in Fig. 1C are representative of at least four experiments.

Measurement of pH in the Nramp2 Compartment—Cells grown to confluence on 175-cm2 flasks with an isolation kit from Roche. The protocol consists of the lysis of the cells with 0.1 M Tris buffer, 0.3 M LiCl, 10 mM EDTA, 1% lithium dodecyl sulfate, 5 mM dithiothreitol, pH 7.5, followed by the addition of biotin-labeled oligo(dT)20, followed by capture with streptavidin-coated magnetic particles. After several washes of the beads with 10 mM Tris buffer, 0.2 M LiCl, 1 M EDTA, pH 7.5, the mRNAs were eluted in water heating for 2 min at 65 °C. A first strand cDNA synthesis kit from Roche was used for RT. 0.1 μg of mRNA was used in the RT reaction containing 1 μM dNTP, 5 mM MgCl2, 1.6 μg of oligo(dT)20 primer, 2 μg of random primer pd(N)5, 50 units of RNase inhibitor, and 20 units of avian myeloblastosis virus reverse transcriptase (except where indicated otherwise). Solutions (20 μl) were incubated first at 25 °C for 10 min and then at 42 °C for 60 min. The avian myeloblastosis virus reverse transcriptase was inactivated by incubation at 95 °C. The PCR was then carried out with sets of primers designed to amplify only the desired isoform of Nramp2. The primers were designed based on the alignment of the known sequences of human, rat, and mouse Nramp2 non-IRE or IRE cDNA. The sense primer (5’-GTGGGGATCCCTGCTGTCG-3’, nucleotides 1128–1145) was common for the two sets because it is in a conserved region of both isoforms. The specific antisense primers were chosen in the 3’-region of the coding sequence where IRE and non-IRE differ. The Nramp2-IRE antisense primer was 5’-GGGCTTTGAGATCTTACCG-3’, corresponding to nucleotides 1627–1650, and the Nramp2 non-IRE antisense was 5’-GTTCAAGCAGATGCTTTACCCG-3’ (nucleotides 1654–1674), where R = A or G, K = G or T, M = A or C, and V = A or C or G. The PCR mixture contained 5 μl of cDNA (except in the control without cDNA), 0.2 mM dNTP, 0.2 μg of sense primers (Nramp2 sense), and 0.2 μg of either Nramp2-IRE or non-IRE antisense primers, 5 units of Taq DNA polymerase (New England Biolabs). The reaction was carried out for 30 cycles each of 30 s at 95 °C, followed by 1 min at 58 °C and finally 1 min at 72 °C. To control for specificity of the primer sets, the PCRs

Internal calibration curves of fluorescence ratio versus pH were generated by perfusing the cells with a series of K+ solutions of defined pH, all containing 5 μM mg/l nilepticin.

Reverse Transcription (RT)-PCR—mRNA was prepared from cells grown to confluence on 175-cm2 flasks with an isolation kit from Roche. The protocol consists of the lysis of the cells with 0.1 M Tris buffer, 0.3 M LiCl, 10 mM EDTA, 1% lithium dodecyl sulfate, 5 mM dithiothreitol, pH 7.5, followed by the addition of biotin-labeled oligo(dT)20, followed by capture with streptavidin-coated magnetic particles. After several washes of the beads with 10 mM Tris buffer, 0.2 M LiCl, 1 M EDTA, pH 7.5, the mRNAs were eluted in water heating for 2 min at 65 °C. A first strand cDNA synthesis kit from Roche was used for RT. 0.1 μg of mRNA was used in the RT reaction containing 1 μM dNTP, 5 mM MgCl2, 1.6 μg of oligo(dT)20 primer, 2 μg of random primer pd(N)5, 50 units of RNase inhibitor, and 20 units of avian myeloblastosis virus reverse transcriptase (except where indicated otherwise). Solutions (20 μl) were incubated first at 25 °C for 10 min and then at 42 °C for 60 min. The avian myeloblastosis virus reverse transcriptase was inactivated by incubation at 95 °C. The PCR was then carried out with sets of primers designed to amplify only the desired isoform of Nramp2. The primers were designed based on the alignment of the known sequences of human, rat, and mouse Nramp2 non-IRE or IRE cDNA. The sense primer (5’-GTGGGGATCCCTGCTGTCG-3’, nucleotides 1128–1145) was common for the two sets because it is in a conserved region of both isoforms. The specific antisense primers were chosen in the 3’-region of the coding sequence where IRE and non-IRE differ. The Nramp2-IRE antisense primer was 5’-GGGCTTTGAGATCTTACCG-3’, corresponding to nucleotides 1627–1650, and the Nramp2 non-IRE antisense was 5’-GTTCAAGCAGATGCTTTACCG-3’ (nucleotides 1654–1674), where R = A or G, K = G or T, M = A or C, and V = A or C or G. The PCR mixture contained 5 μl of cDNA (except in the control without cDNA), 0.2 mM dNTP, 0.2 μg of sense primers (Nramp2 sense), and 0.2 μg of either Nramp2-IRE or non-IRE antisense primers, 5 units of Taq DNA polymerase (New England Biolabs). The reaction was carried out for 30 cycles each of 30 s at 95 °C, followed by 1 min at 58 °C and finally 1 min at 72 °C. To control for specificity of the primer sets, the PCRs
were also performed on 10 ng of Nramp2-IRE or Nramp2 non-IRE plasmids. The products were then separated by electrophoresis in 0.8% agarose.

RESULTS

Expression of Nramp2 Isoforms in LLC-PK₁ Cells—LLC-PK₁ cells, derived from the porcine proximal tubule, have been used extensively as a model of epithelial transport and polarity. To ascertain whether these cells represent a valid model to analyze the subcellular distribution of isoform II in epithelial cells, for comparison with nonepithelial cells, we first studied the expression of Nramp2 isoforms by RT-PCR. Isoform-specific primers were designed from the distinctive region at the 3′-end of the coding sequence of the two variants. To confirm the specificity of the primers, we performed PCR using plasmids encoding either isoform I (IRE) or II (non-IRE). As shown in Fig. 1A, the IRE primer set amplified a fragment of the predicted size, 515 bp, only when the corresponding plasmid was used, but not when the non-IRE plasmid was present. Conversely, only the non-IRE (isoform II) plasmid supported amplification of the predicted 540-bp product when the non-IRE primer was added. Positive identification of the isoforms was also obtained when they were heterologously expressed in LLC-PK₁ cells by stable transfection of the corresponding cDNA (lower panel in Fig. 1A). Having validated the specificity of the primers, we proceeded to define the isoforms expressed by LLC-PK₁ cells. As shown in Fig. 1, both the IRE and non-IRE primers yielded positive bands of the predicted size. In both instances, bands of slightly lower mobility were also detected, possibly reflecting a splicing intermediate. The specificity of these bands, as well as of those of the predicted size, was validated by omission of the reverse transcriptase or of template (Fig. 1A). These observations indicate that both the IRE and non-IRE forms of Nramp2 coexist in LLC-PK₁ cells, which are therefore a suitable model system to study either variant of the transporter.

Expression and Functional Characterization of Nramp2-HA—Antibodies that recognize the individual Nramp2 isoforms are currently not available, nor are there antibodies to the extracellular domain of the proteins that could be used to study their traffic in live cells. To overcome this limitation, we generated a construct encoding a tagged form of isoform II bearing a HA epitope, called Nramp2-HA hereafter. The HA tag was inserted at position 345, in a predicted external (extracytoplasmic) loop between transmembrane domains 7 and 8 of the non-IRE variant of the transporter (see Fig. 1B), enabling us to label plasmalemmal Nramp2 in intact cells. This construct, inserted into the pCB6-neo vector, was used to transfect LLC-PK₁ cells, and stable clones were isolated after neomycin (G418) resistance selection.

Expression of Nramp2-HA was tested by immunoblotting, and the clones showing the highest levels of expression were selected for further study. As shown in Fig. 1C (Control lane), three immunoreactive species were detectable in cells expressing Nramp2-HA: a minor protein of 65 kDa (band b), a second protein of intermediate abundance of 71 kDa (band c), and a major component of ~90 kDa which migrated as a wide band (d), likely attributable to heterogeneous glycosylation. This assumption was validated by two observations. First, treatment of the cells with an N-glycosylation inhibitor (tunicamycin, 2.5 μg/ml for 24 h) resulted in the virtual disappearance of the higher molecular mass bands c and d, with the concomitant appearance of a major ~60-kDa polypeptide (band a in Fig. 1C, middle lane), likely representing the nonglycosylated form of Nramp2. Second, exposure of mature Nramp2 extracted from untreated cells to peptide N-glycanase F, an enzyme that cleaves N-glycosylation sites, similarly led to the disappearance of the heterogeneous ~90-kDa band with generation of ~60-kDa species. Jointly, these observations indicate that, like the wild-type parental protein (18), Nramp2-HA is modified extensively post-translationally by N-linked glycosylation.

We next verified whether the protein tagged with the exofacial epitope retained its functional properties. It had been demonstrated earlier that Nramp2 mediates the transport of several divalent cations, including Co²⁺, across the plasma membrane (5, 12). We therefore measured the rate of Co²⁺ uptake in cells loaded with the metal-sensitive dye calcein, which can be loaded into intact cells as the membrane-permeant acetoxymethyl ester precursor. The anionic form of calcein, which is released intracellularly by deesterification of the precursor, undergoes fluorescence quenching upon binding of Co²⁺, thereby serving to monitor the uptake of the cation. As illustrated in Fig. 1D, untransfected LLC-PK₁ cells are poorly permeant to Co²⁺ at both neutral and acidic pH, as shown by the invariant fluorescence of calcein after exposure of the cells to 50 μM extracellular Co²⁺. By contrast, rapid quenching of calcein was noted upon addition of the metal to cells trans-
fected with Nramp2-HA. Uptake of \( \text{Co}^{2+} \) was faster at acidic pH, consistent with the proposal that Nramp2 functions as a proton:metal symporter.

Subcellular Localization of Nramp2-HA—We next determined the subcellular distribution of Nramp2-HA. To distinguish plasmalemmal from intracellular transporters, the cells were exposed to anti-HA antibodies either before (Fig. 2, A and D) or after permeabilization (Fig. 2, B, C, E, and F). As shown in Fig. 2A, Nramp2-HA was detected in the plasmalemma of LLC-PK\(_1\) cells in a nonuniform, punctate distribution reminiscent of microvilli. This distribution is not unique to LLC-PK\(_1\) cells because a very similar pattern was observed in CHO cells transfected with the same construct (Fig. 2D). A fraction of the transporters was intracellular and thus only apparent after permeabilization of the plasma membrane. In permeabilized LLC-PK\(_1\) and CHO cells, a subpopulation of Nramp2-HA was located in a vesicular/tubular compartment which accumulated preferentially in the juxtanuclear region (Fig. 2, B and E). This bimodal distribution was not artifactually introduced by the extracellular HA epitope because a similar pattern was discerned in LLC-PK\(_1\) and CHO cells stably transfected with Nramp2 tagged at its C terminus with the Myc epitope tag (for location of tag, see diagram in Fig. 1B; and for immunostaining images, see Fig. 2, C–F). The presence of Nramp2 at both the cell surface and in endomembranes had been reported for the endogenous protein (23), further validating the use of the externally tagged construct for studies of intracellular traffic.

We proceeded to identify the intracellular compartment where Nramp2-HA is located, using dual labeling with HA antibodies and markers of specific organelles. As illustrated in Fig. 3, there was no significant overlap between the Nramp2-HA and markers of the Golgi complex (\( \alpha \)-mannosidase II, Fig. 3, E and F) or of late endosomes/lysosomes (Rab7, Fig. 3, G and H) and only partial overlap with markers of the endoplasmic reticulum (KDEL-GFP, Fig. 3, A and B). In contrast, extensive overlap with markers of early/recycling endosomes was noted (syntaxin 13, Fig. 3, C and D; transferrin receptors, Fig. 4). Similar results were obtained in LLC-PK\(_1\) and CHO cells. We concluded that the major site of intracellular expression of isofrom II of Nramp2 in both epithelial (LLC-PK\(_1\)) and non epithelial (CHO) cells is the early/ recycling endosome.

Quantification of the Plasmalemmal and Endomembrane Fractions of Nramp2—The size of the individual cellular pools of Nramp2 (i.e. plasma membrane, endosomes and endoplasmic reticulum) was quantified using \( ^{125} \text{T} \)-labeled antibodies. We initially determined the time required for saturation of the available epitopes by the primary antibody, under the conditions used. As illustrated by the inset in Fig. 4J, binding to the exposed epitopes was very rapid, reaching completion within 30 min at 4 °C. Intact cells were then fixed and exposed to the antibody under saturating conditions in the cold. After washing, the cells were exposed to radiolabeled secondary antibody for quantitation of plasmalemmal Nramp2-HA. In parallel, an equivalent number of cells was initially fixed and permeabilized, then exposed to the primary and secondary antibodies for determination of total Nramp2-HA content. Ten such measurements revealed that 34.9 ± 5.9% (mean ± S.E.) of the total Nramp2-HA resides at the cell surface. The remaining 65% must therefore be found in early/recycling endosomes and/or in the endoplasmic reticulum.

Quantitation of the Recycling Compartment—To define the relative proportion of these compartments, we tried to estimate the size of the recycling compartment. We hypothesized that, unlike the fraction in the endoplasmic reticulum, the endosomal compartment would be in rapid equilibrium with the plasmalemmal Nramp2-HA. To access this dynamic compartment, we carried out labeling of Nramp2-HA on live cells. Extensive cross-linking of membrane proteins by antibodies can alter their subcellular distribution and traffic. For this reason, we used a monoclonal antibody that would, at most, generate dimers. Nevertheless, it was important to verify that such dimerization would not alter Nramp2 dynamics. To this end, we compared the distribution of Nramp2-HA when tagging the epitope with either intact antibody or with a Fab fragment of the anti-HA antibody. After a defined period of internalization, the cells were fixed, permeabilized, and the distribution of Nramp2-HA was revealed by secondary antibodies. As illustrated in Fig. 4, the labeling pattern was identical whether the monovalent fragment or the bivalent antibody was used. Similar results were obtained at different times of internalization, indicating that, if it was occurring, dimerization had no effect on the kinetics of Nramp2 traffic. Because labeling was more robust using the complete antibody, these conditions were used subsequently.

When surface Nramp2 molecules were labeled with anti-HA

**Fig. 3.** Comparison of Nramp2 localization with organellar markers. LLC-PK\(_1\)-Nramp2-HA cells were fixed, permeabilized, and stained with anti-HA antibodies (A, C, E, and G). In B, D, and H the cells had been transfected previously with GFP-tagged organellar indicators, although in F they were immunostained with antibodies to \( \alpha \)-mannosidase II, a Golgi marker. A and B, cells transfected with GFP-KDEL, an endoplasmic reticulum marker; C and D, cells transfected with GFP-syntaxin 13, an early endosomal marker; G and H, cells transfected with GFP-Rab7, a late endosome/lysosome marker. Insets show magnifications of the area boxed in the figure. Images are representative of at least three experiments of each type.
antibody, their rate of disappearance could be quantified by adding the secondary radiolabeled antibody at increasingly longer intervals. As shown in Fig. 4F, about 50% of the exposed Nramp2-HA was internalized with a half-time of 15 min (equivalent to a rate constant of 0.045 min⁻¹). It is unclear from these experiments whether the 50% plateau reached indicates that only a fraction of the plasmalemmal Nramp2 is mobile or that rapid recycling from the cell interior returns a fraction of the labeled transporters to the surface at longer times.

As shown in Fig. 4, C–H, the antibody-associated Nramp2 that disappears from the plasma membrane enters the early/recycling endosomal compartment. There is a progressive accumulation of the internalized antibody in a compartment which colocalizes perfectly with transferrin receptors, without staining of the endoplasmic reticulum. Based on these observations, we were able to quantify the relative size of the recycling compartment. Cells were exposed to HA antibody at 37 °C for varying times, then fixed, permeabilized, and incubated with radiolabeled secondary antibody. These results were normalized to the total Nramp2 content, determined as above by first fixing and permeabilizing the cells, prior to addition of the primary antibodies. As illustrated in Fig. 4J about 30% of Nramp2 is labeled very rapidly under these conditions, likely reflecting the pool of exofacial transporters. This is consistent with the earlier determinations of surface-exposed transporters obtained in the cold, which yielded a value of 34.9%. A second component equilibrates more gradually, with a half-life of ~25 min, consistent with internalization to the endosomal compartment. After 3 h, the uptake reached about 65% of the total available Nramp2. If it is assumed that the entire endosomal compartment is able to recycle within this time frame, and subtracting the contribution of the plasmalemma, we conclude that the recycling endomembrane compartment comprises 30–35% of the total cellular Nramp2.

Role of Clathrin and Dynamin in Nramp2 Endocytosis—The mechanism underlying the internalization of isoform II was investigated next. We tested whether, like the transferrin receptor that is also routed to recycling endosomes, Nramp2-HA undergoes endocytosis via clathrin-coated pits. To this end, LLC-PK₁-Nramp2-HA cells were transfected transiently with a
clathrin light chain-GFP chimera. The localization of this chimera was then compared with that of exocytic Nramp2, visualized by adding anti-HA and Cy3-conjugated secondary antibodies to intact cells. Fig. 5, A and B, shows that there was partial but reproducible colocalization of Nramp2-HA with sites of clathrin accumulation, suggesting that the metal transporter may utilize coated pits for internalization.

The functional role of clathrin-coated pits in Nramp2-HA endocytosis was tested by transfecting the cells with Dyn-1K44A, an inactive form of dynamin which has been well documented to exert a dominant-negative effect on clathrin-mediated internalization (32, 33). The recycling of Nramp2 was then assessed as described for Fig. 4. Although cells not transfected with Dyn-1K44A accumulated labeled Nramp2 in recycling endosomes, cells expressing the dominant-negative construct had little or no internalized Nramp2 (Fig. 5, C and D).

Although these findings seem to implicate clathrin-coated pits in the uptake of Nramp2, recent evidence indicates that dynamin is also involved in the formation of caveolae (34). We considered this possibility by disrupting the lipid rafts that constitute caveolae, using methyl-β-cyclodextrin (35). Cells treated with or without this cholesterol-extracting reagent were used for determinations of the rate of internalization, using the radiolabeled method detailed in Fig. 4. The results of these experiments, summarized in Fig. 5E, clearly indicate that Nramp2 internalization does not require the integrity of rafts and is therefore unlikely to be mediated by caveolae. We therefore conclude that clathrin-mediated endocytosis is the most likely mechanism of Nramp2 internalization.

Role of Phosphatidylinositol 3-Kinase in Nramp2 Recycling—Generation of phosphatidylinositol 3-phosphate by phosphatidylinositol 3-kinase (PI3K) is important for several aspects of early endosomal traffic (36–38). It was therefore of interest to define whether Nramp2 recycling is similarly dependent on normal PI3K activity. This was evaluated using wortmannin, a potent and irreversible inhibitor of class I and III PI3K (36). As illustrated in Fig. 5E, the rate of recycling of Nramp2-HA was markedly depressed by pretreatment with wortmannin. Direct visualization of the intracellular Nramp2-containing compartment by immunostaining of permeabilized cells (Fig. 6D) demonstrated an enlargement of the Nramp2-containing vesicles and the occurrence of tubulation that were not apparent in the control (Fig. 6A).

To define better the site and mode of action of wortmannin on Nramp2, we measured the proportion of the transporter at the surface membrane, using isotopic determinations. Fig. 6C shows that inhibition of PI3K was accompanied by a pronounced decrease in plasmalemmal transporters, from 34.9% ± 5.9 of the total to 17.1% ± 3.0 (means ± S.E. of six experiments).

Additional insight into the mechanism of action of wortmannin was gained by prelabeling exocytic Nramp2 molecules in the cold and then measuring the rate of internalization after rewarming. As shown earlier, in untreated cells only ~50% of Nramp2 disappears from the plasmalemma, even after long incubations, likely resulting from the reappearance of internalized molecules at the surface caused by recycling. By contrast, a much larger fraction of Nramp2 became internalized in wortmannin-treated cells (Fig. 6D). These findings imply that endocytosis of the transporters is not impaired by inhibition of PI3K (the rate constant of internalization was 0.09 min⁻¹, which compares favorably with that determined earlier for the controls). Instead, the findings are consistent with a model in which derangement of the structure and function of the recycling compartment reduces the rate of delivery of internalized Nramp2 to the membrane. Reduced recycling, in combination with active internalization, results in a net decrease of the density of Nramp2 at cell surface, as detected in Fig. 6C.

Earlier studies had reported that treatment with wortmannin decreased the density of transferrin receptors at the surface by ~50%, whereas increasing their rate of endocytosis by up to 60% (39). As concluded above for Nramp2, it was found that inhibition of PI3K depressed the rate of recycling of transferrin receptors (37, 39, 40). The similarities in the intracellular traffic of transferrin and isoform II of Nramp2 are consistent with a close functional relationship between these molecules.

Determination of the pH in Nramp2-expressing Endosomes—Nramp2 has been proposed to operate as a divalent metal-proton symporter (5). The rate of metal transport is therefore predicted to be strictly dependent on the prevailing pH. Although the purported function of Nramp2, namely catalyzing
net metal entry into the cells, would be greatly facilitated by an acid extracellular environment, the extracellular pH is regulated stringently at a moderately alkaline level. On the other hand, the lumen of endosomes is topologically equivalent to the extracellular space, and, as quantified above, a large fraction of Nramp2 exists in endomembranes. We therefore proceeded to measure the luminal pH of the compartments where Nramp2-HA is present. Although recycling endosomes had been reported to be moderately acidic, this compartment is known to be heterogeneous, composed of distinct subcompartments that may vary in pH.

To measure exclusively the pH of the Nramp2 compartment, cells expressing the transporter tagged with the exofacial epitope were incubated with the mouse anti-HA antibody followed by a fluorescein isothiocyanate-conjugated Fab fragment of anti-mouse antibody. Fab fragments of the secondary antibody were used to prevent Nramp2 clustering and possible mistargeting. After incubation at 37 °C to allow for internalization of the labeled complexes, we took advantage of the pH sensitivity of the fluorescein moiety to monitor pH by ratio imaging (for a description of the strategy, see Fig. 7A). The summary of multiple measurements is presented in Fig. 7B. The basal pH of the Nramp2 compartment was found to be ~6.2. Accordingly, the addition of ammonium induced a marked alkalosis, which was readily reversed upon removal of the weak base. The acid pH was maintained by a vacuolar-type H⁺ pump, because it was rapidly dissipated upon addition of nM concentrations of the specific inhibitor folimycin. These observations also indicate that the Nramp2 endosomes have a large "leakage" permeability toward H⁺. The presence of isoform II in an acidic intracellular compartment is compatible with a role in proton-driven delivery of iron to the cytosol.

**Fig. 6. Effect of wortmannin on the recycling of Nramp2.** A and B, effect of wortmannin on the morphology of the Nramp2 compartment. LLC-PK₁-Nramp2-HA cells were incubated for 1 h in the absence (A) or presence (B) of 100 nM wortmannin. The cells were next fixed, permeabilized, and stained with mouse anti-HA antibody followed by Cy3-coupled goat anti-mouse antibody. Results are representative of three experiments. C, effect of wortmannin on the surface expression of Nramp2. LLC-PK₁-Nramp2-HA cells were incubated for 1 h in the absence (solid bar) or presence (open bar) of 100 nM wortmannin. The surface density of Nramp2 was then quantified by equilibrating intact (nonpermeabilized) cells with anti-HA antibody followed by a radiolabeled secondary antibody. D, LLC-PK₁-Nramp2-HA cells were incubated for 1 h in the absence (solid symbols) or presence (open symbols) of 100 nM wortmannin. The rate of Nramp2 internalization was then measured as in Fig. 4H. Data in C and D are the means ± S.E. of at least four independent experiments.


**DISCUSSION**

The IRE-containing Nramp2 was initially found to be the predominant variant in epithelial cells. Conversely, the non-IRE form was found to be enriched in other cells, particularly of hemopoietic origin. It is becoming increasingly clear, however, that the expression of the two variants is not mutually exclusive and that the IRE and non-IRE forms coexist in a variety of cells in different proportions. Indeed, the two forms were found to be expressed with nearly identical abundance in two erythroleukemic lines (24). Conversely, the non-IRE isoform was also found to be highly expressed in mouse kidney (26) and lung epithelial cells (41) and also in epithelial cell lines including HEK293 cells and Caco-2 cells (25). In this regard, the finding that LLC-PK1 cells express isoform II is not unexpected and validates the use of this line as a model to study the biology of this splice variant.

Our experiments demonstrated that the majority, if not all the Nramp2 isoform II recycles actively from the plasma membrane to early endosomes. Based on the rate constant, we calculate that about 9% of the surface transporters are internalized per min, and, because the system is at steady state, an equivalent amount must be recycled from the endomembrane compartment to the surface. In our stable transfectants, the plasmalemmal and endosomal compartments comprise 65–70% of the transporters, with the remaining likely located in the endoplasmic reticulum. These conclusions are consistent with the observation that a significant fraction of the protein is incompletely glycosylated (Fig. 1C), in all likelihood reflecting those molecules trapped in the endoplasmic reticulum. If present, the amount of isoform II in late endosomes or lysosomes is minute because we failed to detect significant colocalization with Rab7 (Fig. 3, G and H) or the mannose 6-phosphate receptor (data not shown). In this regard, isoform II would seem to differ from isoform I, which Tabuchi et al. (24) recently reported to accumulate in late endosomes/lysosomes. However, our own unpublished observations suggest that, although partly present in a Rab7-positive compartment, isoform I nevertheless overlaps substantially with isoform II in early endosomes. Indeed, one would anticipate that the plasmalemmal isoform I of Nramp2 would reside, at least temporarily, in early endosomes en route to the late endosome. A precise quantitation of the abundance of isoform I in the individual subcellular compartments is not currently available.

Our results strongly support the notion that isoform II is the entity responsible for transmembrane transport of the iron released from transferrin to the early endosomal lumen. Not only do the transporters and the transferrin receptors coexist in the same endomembrane compartment, but the pH of the lumen of this compartment is conducive to both the release of the metal from apotransferrin and to its efficient uptake across the endosomal membrane. In mkk/mkk mice, which lack a functional Nramp2, receptor-mediated delivery of iron to the lumen of the endosomes would be normal, but its translocation across the endosomal membrane would be impaired, consistent with the observations of Canonne-Hergaux et al. (21). The functional coupling between isoform II and the transferrin receptor and the extensive similarities in their subcellular localization and traffic reported here raise the possibility that these two proteins may be physically associated, an aspect that is currently under investigation.

Although the function of isoform II in early endosomes is apparent, the role of isoform I in late endosomes/lysosomes is less clear. Free iron is unlikely to reach these compartments as it would be effectively taken up at the membrane of epithelia by plasmalemmal Nramp2, and fluid phase delivery to late endosomes/lysosomes would be very inefficient. Also, because iso-
form II coexists with I in epithelia, little transferrin-derived iron would escape early/recycling endosomes to be transported by late endosomes/lysosomes, as proposed in Ref. 24. Instead, it is conceivable that late endosomal/lysosomal isoform I is intended to retrieve metals bound to proteins or other ligands taken up by receptor-mediated endocytosis. In this regard, it is noteworthy that several metal-binding proteins have been reported to be effectively internalized by megalin/cubilin-mediated endocytosis at the apical membrane (42, 43). It is conceivable that isoform I plays a role in the absorption of metals released during the degradation of such internalized proteins.

In conclusion, our data indicate that isoform II of Nramp2 is found predominantly in three compartments: the plasma membrane, early/recycling endosomes, and the endosomal reticulum. The last compartment is clearly the site of biosynthesis of Nramp2. It is conceivable that late endosomal/lysosomal isoform I is in- tended to retrieve metals bound to proteins or other ligands.
