NRAMP2 (natural resistance-associated macrophage protein 2/DMT1 (divalent metal transporter 1)) is a divalent metal transporter conserved from prokaryotes to higher eukaryotes that exhibits an unusually broad substrate range, including Fe²⁺, Zn²⁺, Mn²⁺, Cu²⁺, Cd²⁺, Co²⁺, Ni²⁺, and Pb²⁺, and mediates active proton-coupled transport. Recently, it has been shown that the microcytic anemia (mk) mouse and the Belgrade (b) rat, which have inherited defects in iron transport that result in iron deficiency anemia, have the same missense mutation (G185R) in Nramp2. These findings strongly suggested that NRAMP2 is the apical membrane iron transporter in intestinal epithelial cells and the endosomal iron transporter in transferrin cycle endosomes of other cells. To investigate the cellular functions of NRAMP2, we generated a polyclonal antibody against the N-terminal cytoplasmic domain of human NRAMP2. The affinity-purified anti-NRAMP2 N-terminal antibody recognized a 90–116-kDa membrane-associated protein, and this band was shifted to 50 kDa by deglycosylation with peptide-N-glycosidase F. Subcellular fractionation revealed that NRAMP2 co-sedimented with the late endosomal and lysosomal membrane proteins and LAMP-1 (lysosome-associated membrane protein 1), but not with the transferrin receptor in early endosomes. The intracellular localization of endogenous NRAMP2 and recombinant green fluorescent protein (GFP)-NRAMP2 was examined by immunofluorescence staining and by native fluorescence of GFP, respectively. Both endogenous and GFP-NRAMP2 were detected in vesicular structures and were colocalized with LAMP-2, but not with EEA1 (early endosome antigen 1) or the transferrin receptor. These results indicated that NRAMP2 is localized to the late endosomes and lysosomes, where NRAMP2 may function to transfer the endosomal free Fe²⁺ into the cytoplasm in the transferrin cycle.

Iron is indispensable for life, serving as a metal cofactor for many enzymes, including both non-heme and heme proteins.

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**Human NRAMP2/DMT1, Which Mediates Iron Transport across Endosomal Membranes, Is Localized to Late Endosomes and Lysosomes in HEP-2 Cells**

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The normal human adult has 35–45 mg of iron/kg of body weight. More than two-thirds of the body’s iron content is incorporated into hemoglobin in developing erythroid precursors and mature red cells (1). Nutritional iron absorption (both heme and non-heme iron) occurs primarily in the intestine. Heme iron constitutes only a small fraction of the available dietary iron, but it is highly available for absorption. On the other hand, the absorption of non-heme iron is low and markedly regulated in the first part of the duodenum, in which the acidic pH promotes solubilization of iron transformed to Fe³⁺ by ferrireductase and ascorbate. In non-intestinal cells, iron is taken into the cell by receptor-mediated endocytosis by transferrin (Tf). Specific receptors (Tf receptor (TfR)) on the outer face of the plasma membrane bind diferric Tf with high affinity (2). Once internalized into the cells, the Tf/TfR complex is delivered to endosomes, which are acidified to pH 5.5–6.0 through the action of an ATP-dependent proton pump. Endosomal acidification weakens binding of iron to Tf and produces conformational changes in both Tf and TfR, strengthening their association (3, 4). The apo-Tf/TfR complex is recycled back to the plasma membrane, where apo-Tf is discharged, thereby completing an elegant and efficient cycle. Previously, it was not clear how iron exits from the transferrin cycle endosomes. However, recent studies have provided new insight into this process and demonstrated a surprising link between the Tf cycle and intestinal iron absorption.

Several inbred strains of rodents have functional defects in iron uptake and transport that define obligate intracellular steps in the iron metabolic pathway (5). Among these animals, the microcytic anemia (mk) mouse and the Belgrade (b) rat have autosomal recessive defects in iron metabolism that are associated with defects in erythroid iron utilization and intestinal iron uptake (6–8). Moreover, studies in vivo have shown that this anemia cannot be corrected by increased dietary iron (9, 10) or by direct iron injection (5), suggesting also a second block in iron uptake by red blood cell precursors and other peripheral tissues. Using a positional cloning approach to identify the gene defective in these rodents, it has recently been demonstrated that the Nramp2 gene is mutated in both the mk

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1 The abbreviations used are: Tf, transferrin; TfR, transferrin receptor; NRAMP, natural resistance-associated macrophage protein; DMT1, divalent metal transporter 1; pAb, polyclonal antibody; mAb, monoclonal antibody; GST, glutathione S-transferase; EA1, early endosome antigen 1; LAMP, lysosome-associated membrane protein; PNS, post-nuclear supernatant; PBS, phosphate-buffered saline; PNGase F, peptide N-glycosidase F; PFA, p-formaldehyde; GFP, green fluorescent protein.
and b animal models (11, 12). Indeed, both the m<sub>e</sub> mouse and the b rat have been shown to carry the same mutation at N<sub>ramp2</sub>, a glycine-to-arginine substitution (G185R) in one of the predicted transmembrane domains (TM4) of the protein. Using a Xenopus oocyte expression cloning assay to screen for iron uptake, Gunshin et al. (13) searched for an intestinal iron transporter in the duodenal mRNA from rats fed a low-iron diet. A single cDNA (initially named Dct1 (divalent cation transporter 1), but recently renamed as Dmt1 (divalent metal transporter 1)) encoding the rat isoform of N<sub>ramp2</sub> was found to stimulate iron uptake by ~200-fold. Voltage-clamp analysis of N<sub>ramp2</sub>Dmt1-mediated iron transport indicated a pH-dependent electrogenic process, similar to those of other transmembrane transporters. A variety of other ions, including Zn<sup>2+</sup>, Mn<sup>2+</sup>, Cu<sup>2+</sup>, Cd<sup>2+</sup>, Co<sup>2+</sup>, Ni<sup>2+</sup>, and Pb<sup>2+</sup>, stimulated currents indistinguishable from that of iron at the same concentration, suggesting that N<sub>ramp2</sub>Dmt1 can transport a variety of divalent metal ions. N<sub>ramp2</sub> is an orphan protein with no known function cloned based on its homology to N<sub>ramp1</sub> (14). N<sub>ramp1</sub> has been identified in the mouse Bcg<sub>III</sub>Lsh locus by positional cloning, which controls resistance to infection in vivo with Mycobacterium, Salmonella, and Leishmania (15). The N<sub>ramp1</sub> and N<sub>ramp2</sub> proteins are highly similar (64% overall identity) and are highly hydrophobic integral membrane glycoproteins composed of 12 transmembrane domains that possess several structural characteristics of ion channels and transporters. The members of the mammalian N<sub>ramp</sub> gene family are homologous to the yeast S<sub>mpf</sub> gene family of Mn<sup>2+</sup> transporters (16). Mouse N<sub>ramp2</sub> and human N<sub>ramp2</sub> can functionally complement a Saccharomyces cerevisiae s<sub>mpf1</sub>s<sub>mpf2</sub> null mutant and a Schizosaccharomyces pombe p<sub>dtt1</sub> null mutant, respectively, whereas mouse N<sub>ramp1</sub> and human N<sub>ramp1</sub> cannot (17, 18). The functions of N<sub>ramp1</sub> are still unknown, but this molecule is predicted to function as a metal transporter similar to N<sub>ramp2</sub>. As opposed to N<sub>ramp1</sub>, which is expressed exclusively in mononuclear phagocytes such as tissue macrophages (15), N<sub>ramp2</sub> mRNA expression is more ubiquitous and has been detected in most tissues and cell types analyzed (13, 14, 19). However, its levels of expression are higher in the brain, thymus, proximal intestine, kidney, and bone marrow (13). Immunocytochemical analysis with protein-specific antibodies revealed that N<sub>ramp1</sub> is expressed in the late endosomal and lysosomal membranes in macrophages and is recruited to the membrane of the phagosome upon phagocytosis (20–22). On the other hand, some groups argued that N<sub>ramp2</sub> is expressed in the apical membrane in the brush border of intestinal enterocytes and in TI-positive recycling endosomes in non-intestinal cells (23–25). In previous studies, we have reported both cDNA and genomic DNA structures of human N<sub>ramp2</sub> (26, 27), generated human N<sub>ramp2</sub> N-terminal domain-specific antisense, and showed that this antisense functionalized recognition recombinant N<sub>ramp2</sub> protein expressed in fission yeast (18). In this study, we investigate the subcellular localization of N<sub>ramp2</sub> in cultured human cells with the affinity-purified anti-N<sub>ramp2</sub> N-terminal antibody and showed that N<sub>ramp2</sub> is localized to late endosomes and lysosomes.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Production of anti-N<sub>ramp2</sub> N-terminal antisera was described previously (18). The affinity-purified anti-N<sub>ramp2</sub> N-terminal monoclonal antibody (mAb) (N-2) was pre-
pared as described previously (28). Mouse anti-human EEA1 mAb and mouse anti-human LAMP-1 mAb were purchased from Transduction Laboratories. Mouse anti-human LAMP-2 mAb (H4B4, developed by Drs. J. E. K. Hildreth and J. T. August) was obtained from the Developmental Studies Hybridoma Bank (Baltimore, MD). Alexa 594-labeled antirat IgG, anti-mouse IgG, Alexa 488-labeled anti-rabbit IgG, and Texas Red-dextran were purchased from Molecular Probes, Inc. Cy2-labeled anti-mouse IgG was purchased from Jackson ImmunoResearch Laboratories, Inc.

Cell Culture and Transfection—The human larynx carcinoma cell line HEp-2 and human cervix carcinoma cell line HeLa were maintained in Dulbecco’s minimal essential medium (Sigma) containing 10% fetal calf serum, 50 μg/ml penicillin and 50 μg/ml streptomycin. The human green monkey kidney cell line COS-7 was maintained in high-glucose Dulbecco’s minimal essential medium (Sigma) supplemented with 10% fetal calf serum, 50 μg/ml penicillin, and 50 μg/ml streptomycin. FuGENE<sup>TM</sup> 6 transfection reagent (Roche Molecular Biochemicals) was used for the transient transfection of HEp-2, HeLa, and COS-7 cells according to the manufacturer’s instructions.

Vector Construction—The human N<sub>ramp2</sub> cDNA was amplified using the sense primer NR2-1 (5′-TGATCACACCTTGGCTGGTGC-3′, possessing a 5′-BclI site (underlined) and the first six amino acids including the initiation methionine (double-underlined) of N<sub>ramp2</sub>) and the antisense primer NR2-3 (5′-TGATCGTTTATTCATTCG-3′, possessing a 5′-BclI site (underlined) and the final five amino acids and stop codon (double-underlined) of N<sub>ramp2</sub>) and human N<sub>ramp2</sub> cDNA (26) as a template. The reaction product was purified by agarose gel electrophoresis and cloned into the pGEM-T easy vector (Promega). Nucleotide sequences of the polymerase chain reaction products were verified by the dideoxynucleotide chain termination method using a LI-COR 4000L automated DNA sequencer.

The resulting plasmid, pN<sub>ramp2</sub>-2, was prepared from E. coli strain SCS110 (Stratagene) and digested with BclI, and the BclI fragment containing the full-length N<sub>ramp2</sub> cDNA was ligated into the BamHI site of pGFP-C1 (CLONTECH) to generate pGFP-N<sub>ramp2</sub>.

Differential Extraction and Immunoblot Analysis—HEp-2 cells were homogenized with 15 strokes in a Potter homogenizer and centrifuged at 800 × g for 5 min. The post-nuclear supernatant (PNS) was centrifuged for an additional 30 min at 128,000 × g to recover the pellet as the membrane fraction. The membrane fraction was resolved in 100 μl of denaturing buffer (0.5% SDS and 0.1 μM β-mercaptoethanol in PBS) and denatured for 5 min at 95 °C. Protein concentration was determined by the Bradford assay (Bio-Rad). For differential extraction, HEp-2 membrane fraction was suspended in a solution containing NaCl, urea, and N<sub>10</sub>H<sub>4</sub>NO<sub>3</sub>, pH 10.0, at final concentrations of 1.0, 2.0, and 0.1 M, respectively. The suspension was incubated on ice for 30 min and then centrifuged at 128,000 × g for 30 min at 4 °C. The supernatant was transferred to a fresh tube, precipitated with 10% trichloroacetic acid, and resolved in 100 μl of Laemmli sample buffer. The precipitate was dissolved in 100 μl of Laemmli sample buffer. The supernatant (S) and precipitate (P) fractions were resolved by SDS-polyacrylamide gel electrophoresis and immunolabeled using the affinity-purified anti-N<sub>ramp2</sub> N-pAb. For peptide N-glycosidase F (PNGase F) digestion, aliquots of 0.1 μl Na<sub>2</sub>CO<sub>3</sub>-treated membrane fractions prepared from HEp-2 cells and the membrane fraction of recombinant N<sub>ramp2</sub> expressed in fission yeast were denatured before digestion in denaturing buffer for 10 min at 95 °C. PNGase F digestion was performed according to the manufacturer’s instructions (New England Biolabs Inc.). Proteins were transferred onto nitrocellulose membranes, and the blots were incubated with the primary antibodies. Proteins were detected with horseradish peroxidase-conjugated antibody against rabbit IgG (Amerham Pharmacia Biotech).

Subcellular Fractionation—Following two rinses with ice-cold PBS, confluent HEp-2 cells in 450-cm² culture dishes were scraped with a rubber policeman, harvested in ice-cold PBS, and collected by centrifugation at 800 × g for 5 min. Cells were resuspended in 1.0 ml of ice-cold homogenization buffer (0.3 M sucrose and 10 mM HEPES, pH 7.3) and homogenized with 10 strokes in a Potter homogenizer. The cell homogenate was centrifuged at 500 × g for 10 min at 4 °C, and the PNS was saved. The pellet was resuspended in 1.0 ml of ice-cold homogenization buffer, homogenized with 10 strokes, and centrifuged. A total of 2.0 ml of PNS was recovered when then 1.5 ml of 10 mM Na<sub>2</sub>CO<sub>3</sub> and 31 ml of a 0.3–1.7 M preformed linear sucrose gradient in homogenization buffer. The gradients were centrifuged at 24,000 rpm for 3 h at 4 °C in a P28S rotor (Hitachi Co. Ltd.) and then removed in 3-ml fractions from the bottom of the tube. Each fraction was dialyzed with PBS overnight and then centrifuged at 128,000 × g for 30 min to recover the pellet as the membrane fraction. The membrane fractions were resolved.
Localization of NRAMP2

in 100 μl of Laemmli sample buffer, and aliquots of 5 μl were resolved by 10% SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting. The enrichment of proteins was confirmed by densitometric analysis using NIH Image Version 1.59.

Immunofluorescence Microscopy—Cells grown on glass coverslips were washed three times with PBS, fixed with 4% paraformaldehyde (PFA) in PBS for 15 min at room temperature, and permeabilized with 50 μg/ml digitonin in PBS for 10 min. The coverslips were washed twice (10 min/wash) with PBS and blocked in 0.2% fish skin gelatin in PBS for 10 min. Cells were incubated with primary antibodies diluted in PBS for 60 min at room temperature. Coverslips were washed three times (10 min/wash) with 0.2% fish skin gelatin in PBS. Secondary antibodies coupled to Alexa 488, Alexa 594, or Cy2 were diluted in PBS and incubated on coverslips for 60 min at room temperature. Coverslips were washed with 0.2% fish skin gelatin in PBS as described above and mounted on slides in 9:1 glycerol/PBS. Antibodies were used at the following dilutions: affinity-purified anti-NRAMP2 N pAb, 1:50; anti-TR mAb, 1:500; anti-LAMP-2 mAb, 1:1000; anti-EEA1 mAb, 1:50; Alexa 594-labeled anti-rabbit IgG, Alexa 594-labeled anti-mouse IgG, and Alexa 488-labeled anti-rabbit IgG, 1:500; and Cy2-labeled antimouse IgG, 1:100. To label the lysosomal compartment, HEp-2 cells were incubated with 1 mg/ml lysine-fixable Texas Red-dextran in Dulbecco’s minimal essential medium for 3 h at 37°C in 5% CO₂. Cells were stripped with trypsin/EDTA, washed twice with PBS, grown on glass coverslips for 12 h at 37°C in 5% CO₂, and then fixed with 4% PFA. GFP-NRAMP2-transfected cells grown on glass coverslips were treated with nocodazole (100 μg/ml for HEp-2 cells and 20 μg/ml for HeLa and COS-7 cells). After incubation for 1 h at 37°C, cells were washed three times with PBS, fixed with 4% PFA, permeabilized, and then incubated with primary antibodies followed by secondary antibodies. The coverslips were examined with an Olympus BX50 microscope. Photographs were taken with an Olympus color chilled 3CCD camera (Model M-3204C-10). Images were analyzed on a Macintosh computer using Adobe Photoshop 4.0J.

RESULTS

Generation of Antibody to Detect Human NRAMP2—NRAMP2 is an integral membrane protein that consists of 12 putative transmembrane domains and two potential glycosylation sites as shown in Fig. 1A. Previously, we produced a polyclonal antibody against the N-terminal cytoplasmic tail of human NRAMP2 by immunization with recombinant TrpE-NRAMP2-(1–66) protein produced in E. coli and showed that this anti-NRAMP2 N antiserum recognized the functional NRAMP2 protein expressed in fission yeast (18). However, we failed to detect endogenous human NRAMP2 in various human cell lines with this anti-NRAMP2 N antiserum (data not shown). We purified this anti-NRAMP2 N antiserum by recombinant GST-NRAMP2-(1–66) fusion protein-coupled affinity column chromatography to detect the endogenous NRAMP2 protein by immunoblotting and indirect immunofluorescence analysis. Anti-NRAMP2 N antiserum recognized not only the TrpE-NRAMP2-(1–66) fusion protein, but also the TrpE protein (Fig. 1B, panel b), and preimmune serum did not recognize either protein (panel a). The affinity-purified anti-NRAMP2 N pAb recognized only the TrpE-NRAMP2-(1–66) fusion protein, but not the TrpE protein (Fig. 1B, panel c). These results indicated that the affinity-purified NRAMP2 N pAb specifically recognized only the portion of amino acids 1–66 of human NRAMP2. To identify endogenous NRAMP2, immunoblot analysis was performed with the affinity-purified NRAMP2 N pAb using the membrane fractions prepared from HEp-2 and COS-7 cells (Fig. 1C). The affinity-purified anti-NRAMP2 N pAb recognized both the 66-kDa (p66) band and the 90–116-kDa (p116) band on immunoblot analysis of the HEp-2 membrane fraction (Fig. 1C, lane 1). In COS-7 cells, the affinity-purified NRAMP2 N pAb recognized only the p66 band, and not the p116 band (Fig. 1C, lane 2). The predicted molecular mass of NRAMP2 is 61.5 kDa, which is close to that of the p66 band. Gruenheid et al. (24) reported that mouse NRAMP2 is a 90–100-kDa integral membrane protein extensively modified by glycosylation (>40% of the molecular mass); this molecular mass is close to that of the p116 band. To determine which band(s) actually represented the human NRAMP2 protein, HEp-2 membrane fractions were extracted with 1 M NaCl, 2 M urea, or 0.1 M Na₂CO₃, pH 11.0. Although both p66 and p116 were sedimented in the membrane fraction, p66 was readily extracted to the soluble fraction by 1 M NaCl or 0.1 M Na₂CO₃ treatment, but p116 could not be extracted under any conditions used in this study (Fig. 1D). These solubilization profiles suggest that p66 is not an integral membrane protein and that p116 could be the NRAMP2 protein. To confirm the identity of p116, the 0.1 M Na₂CO₃-extracted membrane fraction from HEp-2 cells and the membrane fraction from NRAMP2 expressed in fission yeast were treated with PNGase F, followed by electrophoresis and immunoblotting. PNGase F treatment of HEp-2 cell membrane resulted in a shift of the apparent molecular mass of the p116 band from 90–116 to 50 kDa (Fig. 1E, lane 2). PNGase F also converted the 90–60-kDa recombinant NRAMP2 expressed in fission yeast into smaller forms with an apparent molecular mass of 50 kDa (Fig. 1E, lane 1), which was the same molecular mass as the band detected upon PNGase F treatment of the HEp-2 cell membrane. These results showed that the 0.1 M Na₂CO₃-resistant p116 band recognized by this pAb was the NRAMP2 protein itself.

NRAMP2 Is Co-sedimented with Lysosome-associated Membrane Protein—Understanding the localization of NRAMP2 within the cell is critical for understanding the Fe²⁺ transport...
step from the endosomes to the cytoplasm in the Tf cycle. To confirm the subcellular localization of NRAMP2 biochemically, we performed subcellular fractionation of HEp-2 cells. PNS was prepared from HEp-2 cells and fractionated on a 0.3–1.7 M sucrose density gradient, and the distribution of NRAMP2 within the gradient was compared with the early endosomal membrane protein TIR and the late endosomal/lysosomal membrane proteins LAMP-1 and LAMP-2. NRAMP2 immunoreactivity was detected in fractions 4–8 and was enriched in fraction 6, coincident with LAMP-1 (Fig. 2). LAMP-2 also appeared essentially in the same position of LAMP-1, and it is obvious that TIR appeared in fractions 5–9, its peak was detected in fraction 7, and NRAMP2 appeared in a different position from TIR (Fig. 2). We repeated the same experiments, and these differences were consistently seen. Thus, the observations in our subcellular fractionation studies suggested that NRAMP2 was expressed within the late endosomal/lysosomal membranes.

**Localization of NRAMP2 in HEp-2 Cells**—To elucidate the intracellular localization of NRAMP2, we performed indirect immunofluorescence staining of NRAMP2. When HEp-2 cells were fixed with 4% PFA and stained using the affinity-purified anti-NRAMP2 N pAb, vesicular structures in the perinuclear regions were stained (Fig. 3a). These structures were not stained by preimmune serum or non-purified antibody (data not shown) or in the presence of an excess amount of the bacterially expressed GST-NRAMP2(1–66) fusion protein that may have been recognized by the antibody (Fig. 3d). When COS-7 cells were stained with the affinity-purified anti-NRAMP2 N pAb, only the nuclear regions were specifically stained (Fig. 3c). As some of the HEp-2 cells were disrupted to too great an extent by detergent through the immunostaining process, they showed staining of not only the perinuclear vesicles, but also the nuclei (Fig. 3b, arrowheads). Immunoblot analysis showed that the affinity-purified anti-NRAMP2 N pAb recognized only p66, but not p116/NRAMP2 in the membrane fraction of COS-7 cells (Fig. 1, lane 2). This indicated that COS-7 cells did not express p116/NRAMP2 proteins that could be recognized by the affinity-purified NRAMP2 N pAb. As the affinity-purified anti-NRAMP2 N pAb specifically recognized a portion of amino acids 1–66 of NRAMP2, these observations suggested that p66 must be the protein containing the same epitope as the N-terminal domain of NRAMP2 and that is localized to the nucleus. Fortunately, because the antibody could not permeate the nucleus in HEp-2 cells under our immunostaining conditions, only the perinuclear vesicular structures representing NRAMP2 were stained. However, because of the different permeability of antibodies, when HeLa cells were stained with the affinity-purified pAb, most of the cells showed staining of not only the perinuclear vesicles, but also of the nuclei under our immunostaining conditions (data not shown).

To identify the perinuclear vesicular structures stained in HEp-2 cells, the cells were double-stained with anti-EEA1 marker for early (sorting) endosomes; anti-TIR marker for sorting endosomes, recycling endosomes, and plasma membrane; anti-LAMP-2 marker for late endosomes and lysosomes; or Texas Red-dextran-endocytosed for 15 h, a marker of lysosomes. The early endosomal markers EEA1 and TIR were not colocalized with NRAMP2 (Fig. 4, A–F), although a small portion of NRAMP2 in the perinuclear region overlapped with TIR (Fig. 4, D–E). In contrast, most of the puncta of NRAMP2 were completely colocalized with LAMP-2 (Fig. 4, G–I); and furthermore, endocytosed Texas Red-dextran in the perinuclear re-
gion, which was supposed to be a lysosomal marker, appeared to be localized in the same compartment as NRAMP2 (Fig. 4, J–L). These results indicated that NRAMP2 was located in late endosomes and lysosomes.

**Localization of GFP-NRAMP2 in HEp-2, HeLa, and COS-7 Cells**—To confirm the late endosomal and lysosomal localization of NRAMP2 by examining the localization of recombinant GFP-NRAMP2, HEp-2 cells were transfected with pGFP-NRAMP2, fixed with 4% PFA, and stained with antibodies for several organelle markers. Similar to the staining with the affinity-purified anti-NRAMP2 N pAb, GFP fluorescence was also detected in vesicular structures in the perinuclear region in transfected HEp-2 cells (Fig. 5, A, D, G, and J), and these structures were not seen in untransfected cells (arrowheads). GFP-NRAMP2 was completely colocalized with LAMP-2 and partially colocalized with endocytosed Texas Red-dextran, but showed no colocalization with EEA1. In addition, GFP-NRAMP2 partially overlapped with the perinuclear region of TIR staining (Fig. 5, D–F). To eliminate the possibility that the localization of NRAMP2 to late endosomes and lysosomes was limited to HEp-2 cells, we also analyzed the subcellular localization of GFP-NRAMP2 in HeLa and COS-7 cells. HeLa and COS-7 cells were transfected with pGFP-NRAMP2, followed by immunostaining with antibodies to TIR and LAMP-2. Similar to the observations obtained by double staining of GFP-NRAMP2 and organelle markers in HEp-2 cells, GFP-NRAMP2 was colocalized with LAMP-2 in HeLa and COS-7 cells (Fig. 5, D–F) and partially overlapped with TIR in the perinuclear region in HeLa cells (Fig. 5, M–O).

**Nocodazole Treatment Does Not Affect the Colocalization of NRAMP2 and LAMP-2**—Both endogenous NRAMP2 and GFP-NRAMP2 partially overlapped with TIR in the perinuclear region in HEp-2 cells and notably in HeLa cells (Figs. 4 D–F and 5 D–F and M–O). A number of membrane compartments were localized to the perinuclear region of the cell, including the Golgi, recycling endosomes and late endosomes and lysosomes. It was previously proposed that the perinuclear structures marked by TIR represent recycling endosomes (described by Mellman and co-workers (29) as recycling vesicles) and that these structures disperse upon treatment with nocodazole. To more rigorously demonstrate the colocalization of NRAMP2 and LAMP-2, we examined the co-distribution of GFP-NRAMP2 and LAMP-2 in cells in which the microtubule cytoskeleton had been depolymerized with nocodazole. Nocodazole treatment also strongly affected the overall organization of late endosomes and lysosomes. These compartments are randomly scattered throughout the cytoplasm and change to enlarged patched structures following nocodazole treatment (30, 31). As shown in Fig. 6 (A, D, G, J, and M), treatment with nocodazole caused the perinuclear structures of GFP-NRAMP2 to scatter throughout the cytoplasm and to patch to the enlarged vesicles. The colocalization between GFP-NRAMP2 and LAMP-2 was also perfectly observed in these structures in all cell lines used in this study (Fig. 6, H, K, and N). However, the localization of GFP-NRAMP2 and TIR was separated by treatment with nocodazole (Fig. 6, B and E). Essentially the same results were obtained by immunostaining of NRAMP2 in HEp-2 cells (data not shown). These observations indicated that NRAMP2 and LAMP-2 were present in the same compartment because if they were in distinct compartments, they would not necessarily remain colocalized following nocodazole treatment. Finally, we concluded that NRAMP2 was located in late endosomes and lysosomes.

**DISCUSSION**

NRAMP2 is a divalent metal transporter that exhibits an unusually broad range of substrate specificity, including Fe$^{2+}$, Zn$^{2+}$, Mn$^{2+}$, Cu$^{2+}$, Cd$^{2+}$, Co$^{2+}$, Ni$^{2+}$, and Pb$^{2+}$ (13). NRAMP2 has been suggested to function as an endosomal iron transporter in non-intestinal cells and as an apical membrane iron transporter in intestinal enterocytes because the microcytic anemia (mk) mouse and the Belgrade (b) rat, which have in-
Inherited defects in iron transport that result in iron deficiency anemia, have the same missense mutation (G185R) in Nramp2 (11, 12). To obtain information about the relationship between the subcellular localization and the function of NRAMP2/DMT1 in the Tf cycle, we investigated the subcellular localization of human NRAMP2 by subcellular fractionation and immunocytochemical analyses and the tagging of NRAMP2 with GFP in HEp-2 cells. In this study, we showed that NRAMP2 was colocalized with late endosomal and lysosomal membrane protein markers, but not with recycling endosomal and/or sorting endosomal protein markers. Recently, Su et al. (25) reported that both wild-type and mutant G185R forms of epitope-tagged mouse Nramp2 are colocalized with Tf in HEK293T cells. Gruenheid et al. (24) also reported that mouse Nramp2 is colocalized with Tf in recycling endosomes in Chinese hamster ovary and RAW264.7 cells. In our study, partial overlap between NRAMP2 and TIR was seen in the perinuclear region of HEp-2 and HeLa cells, and this overlap was especially marked in HeLa cells. In HeLa, Chinese hamster ovary, and some other cells, recycling endosomes and late endosomes and lysosomes

Fig. 5. Localization of GFP-NRAMP2 in HEp-2, HeLa, and COS-7 cells. HEp-2 (A–L), HeLa (M–R), and COS-7 (S–U) cells expressing GFP-NRAMP2 under the control of the cytomegalovirus promoter were fixed and incubated with anti-EEA1 mAb (B), anti-TIR mAb (E and N), or anti-LAMP-2 mAb (H, Q, and T), followed by incubation with the Alexa 594-labeled anti-mouse IgG antibody (B, E, H, N, Q, and T). Lysosomes of transfected HEp-2 cells were stained with Texas Red-dextran (K). Cellular localization of GFP-NRAMP2 was indicated by native GFP fluorescence (A, D, G, J, M, P, and S). Overlays of A and B, D and E, G and H, J and K, M and N, P and Q, and S and T are shown in C, F, I, L, O, R, and U, respectively. The arrows indicate puncta that are fluoroactive for both NRAMP2 and LAMP-2. The arrowheads indicate the untransfected cells. Bar = 10 μm.
are all clustered around the perinuclear microtubule-organizing center, and this makes it difficult, at the level of microscopy, to resolve these organelles in this region (32, 33). In HEp-2 cells, the recycling compartment has a more widely dispersed tubular distribution (34–36). The difference between the localization of NRAMP2 and that of TfR was clearer in HEp-2 cells (Figs. 4 (D–F) and 5(D–F)). The microtubule-depolymerizing agent nocodazole has been shown to cause vesiculation and dispersal of both Golgi cisternae and early endosomal compartments (37). Nocodazole treatment caused the complete separation of NRAMP2 and TfR, but NRAMP2 remained colocalized with LAMP-2 in various cell lines under these conditions (Fig. 6). These results indicate that the NRAMP2 and LAMP-2 proteins are located on the membranes of the same compartments, consistent with the localization of NRAMP2 on late endosomes and lysosomes. It is difficult from immunofluorescence data to make quantitative estimates of protein localizations. We could not exclude the possibility that sorting endosomes contain some NRAMP2 and that this protein is concentrated in late endosomes and lysosomes as they mature.

In iron metabolism, there are three critical steps in which NRAMP2 may function as an iron transporter: (i) the apical membrane dietary iron transport step in intestinal enterocytes, (ii) the phagosomal iron transport step from phagocytosed red blood cells in reticuloendothelial systems, and (iii) the endosomal iron transport step in the Tf cycle in erythrocytes and other cells. Canonne-Hergaux et al. (23) reported that Nramp2 expression is markedly induced in the proximal portion of the duodenum under conditions of dietary iron deficiency and that Nramp2 is abundantly expressed in the brush border of absorptive epithelial cells of the duodenum. These observations demonstrate that Nramp2 is localized to the apical membrane in brush borders, where Nramp2 functions as an apical membrane dietary iron transporter, and indicate that Nramp2 is sorted to the apical membranes by an as yet unknown sorting mechanism specific to intestinal enterocytes. In reticuloendothelial systems, macrophages play a fundamental role in iron metabolism by phagocytosing effete red blood cells, breaking down their hemoglobin, and recycling iron to transferrin for delivery back to the erythron (1, 38, 39). The bulk of iron used for erythropoiesis has passed through this recycling pathway. In reticuloendothelial macrophages, an iron transporter is needed in the phagosomal membranes to release the phagosomal free iron into the cytoplasm. These cells specifically express Nramp1, which is localized to the late endosomes and lysosomes and which is associated with phagosomal membranes during phagocytosis. Nramp1 was suggested to play a special role in this process (39). If Nramp1 functions in this process, Nramp1-deficient mice would suffer from iron deficiency anemia. However, the Bcg mutant mouse or the Nramp1 knockout mouse has not been reported to show recessive defects in iron absorption or to exhibit severe microcytic hypochromic anemia. These observations indicate that Nramp1 does not function in this process. Gruenheid et al. (24) reported that Nramp2 is also expressed in macrophages and is associ-
ated with the phagosomal membrane during phagocytosis. If NRAMP2 functions in this process, the results obtained in this study concerning the late endosomal and lysosomal localization of NRAMP2 are reasonable. In erythrocytes and other cells, iron absorption is mediated in a Tf(TfR)-dependent manner (2). From the current understanding on iron acquisition via Tf(TfR)-mediated endocytosis (33) and the present results, we propose the schematic model for the function of NRAMP2 in the Tf(TfR)-mediated iron transport process (Fig. 7). In the first step, plasma Fe$^{3+}$-Tf attaches to specific Tf receptors on the cell surface by a physicochemical interaction, not requiring temperature or energy. In a temperature- and energy-dependent manner, the Tf(TfR) complexes are then internalized within clathrin-coated vesicles. Iron is released from the Tf within the endocytic vesicles by a temperature- and energy-dependent process that involves endosomal acidification. Influx of protons into the endosomes occurs via an ATP-dependent proton pump (vacuolar H$^+$-ATPase). A separate function that is also a consequence of pH-dependent conformational change is the release of iron from Tf. At the acidic pH of sorting endosomes, iron is released from Tf, and the resulting apo-Tf remains bound to its receptor in the acidic endosomes (3, 4). The apo-Tf(TfR) complex is rapidly sorted to the recycling endosomes and eventually recycles back to the cell surface. The free Fe$^{3+}$ released to endosomes is reduced to Fe$^{2+}$ on the cis-side of the endosomal membrane probably mediated by oxidoreductase (41). Finally, free Fe$^{2+}$ is transported to late endosomes and lysosomes and is then transported into the cytoplasm by NRAMP2. The mechanism of the passage of material from early sorting endosomes to late endosomes is still obscure. Two general models have been proposed (40, 42, 43). In the vesicle transport model (Fig. 7A), the early endosomes would be stable organelles from which transport vesicles would pinch off to deliver endosomal contents to late endosomes. This would be similar to the vesicle traffic mechanisms that operate between the endoplasmic reticulum and the Golgi apparatus. In this model, because it is impossible that free iron is vectorially sorted to late endosomes, specific iron carriers may be expressed in sorting endosomes. Endosomal free iron would be transported to late endosomes by such a specific iron carrier. In the maturation model (Fig. 7B), the entire contents of the early sorting endosomes would be transformed into late endosomes. In this model, free iron is concentrated in sorting endosomes by the Tf cycle, and early sorting endosomes that concentrated free iron gradually mature to late endosomes with recruitment of the late endosomal components (NRAMP2, vacuolar H$^+$-ATPase, etc.) and with
sorting of the early endosomal recycling receptors (TfR, low density lipoprotein receptor, etc.) to recycling endosomes. Finally, endosomes mature to late endosomes, and then endosomal free iron is transported into the cytoplasm by NRAMP2. Although the mechanism of the pathway from early endosome to late endosome is still obscure, the majority of recent data support the maturation model [33]. Once we take a stand on the vesicle transport model for iron transport mediated by NRAMP2, we have to hypothesize the presence of additional iron carrier protein in the vesicle to incorporate Fe^{2+}. Together with the previous studies, we assume that the maturation model is reasonable at the present time.

In conclusion, we have shown that NRAMP2 is a 90–116-kDa integral membrane glycoprotein and is colocalized with the previous studies, we assume that the maturation model is reasonable at the present time.

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