The Iron Transport Protein NRAMP2 Is an Integral Membrane Glycoprotein That Colocalizes with Transferrin in Recycling Endosomes

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Summary

The natural resistance associated macrophage protein (Nramp) gene family is composed of two members in mammals, Nramp1 and Nramp2. Nramp1 is expressed primarily in macrophages and mutations at this locus cause susceptibility to infectious diseases. Nramp2 has a much broader range of tissue expression and mutations at Nramp2 result in iron deficiency, indicating a role for Nramp2 in iron metabolism. To get further insight into the function and mechanism of action of Nramp proteins, we have generated isoform specific anti-Nramp1 and anti-Nramp2 antisera. Immunoblotting experiments indicate that Nramp2 is present in a number of cell types, including hemopoietic precursors, and is coexpressed with Nramp1 in primary macrophages and macrophage cell lines. Nramp2 is expressed as a 90–100-kD integral membrane protein extensively modified by glycosylation (>40% of molecular mass). Subcellular localization studies by immunofluorescence and confocal microscopy indicate distinct and nonoverlapping localization for Nramp1 and Nramp2. Nramp1 is expressed in the lysosomal compartment, whereas Nramp2 is not detectable in the lysosomes but is expressed primarily in recycling endosomes and also, to a lower extent, at the plasma membrane, colocalizing with transferrin. These findings suggest that Nramp2 plays a key role in the metabolism of transferrin-bound iron by transporting free Fe^{2+} across the endosomal membrane and into the cytoplasm.

Key words: iron • anemia • transport • infection • macrophage

Naturally occurring (1) or experimentally induced (2) mutations at the Nramp1 (natural resistance associated macrophage protein) locus in vivo impair macrophage function and cause susceptibility to infection by intracellular pathogens such as Salmonella, Leishmania, and Mycobacterium in mice. In humans, polymorphic variants at Nramp1 are associated with increased susceptibility to tuberculosis and leprosy (3, 4). Studies in vitro in explanted cell populations have indicated that mutations at Nramp1 affect the ability of the macrophage to restrict the intracellular replication of antigenically unrelated microorganisms. We cloned the Nramp1 gene (5) and showed that its mRNA is expressed abundantly in macrophages (6) and in neutrophils (7) and is inducible in macrophages by exposure to cytokines and bacterial endotoxin (6). Predicted amino acid sequence analysis indicates that Nramp1 has many characteristics of an integral membrane transport protein including 12 putative transmembrane (TM) domains, several predicted N-linked glycosylation sites, and a sequence signature previously identified in a number of eukaryotic and prokaryotic transport proteins (5). In macrophages, direct biochemical studies have shown that Nramp1 is a membrane phosphoglycoprotein of apparent mass 90–110 kD (8), which is expressed in the Lamp1-positive lysosomal compartment (9). Moreover, studies in phagosomes containing either latex beads or intact bacteria have shown that upon phagocytosis, Nramp1 is recruited to the membrane of the phagosome, where it remains during its maturation to phagolysosome (9). These findings suggest that Nramp1 may affect resistance to infection by modulating the intravesicular milieu of the bacterial phagosome.

We have identified a second Nramp gene in mammals, Nramp2, which encodes a protein highly similar to Nramp1 (78% identity over the hydrophobic core) (10). As opposed to the phosphocyte-specific expression of Nramp1, Nramp2 mRNA expression has been detected in most tissues and...
cell types analyzed (10–12). Recently, it was shown that the N ramp gene is mutated (G185R ) in two animal models of iron deficiency, the mk mouse (13) and the Belgrade rat (14). The mk mouse displays deficiency in intestinal iron uptake and microcytic anemia (15, 16). The Belgrade rat also shows a defect in intestinal iron absorption (17). Moreover, studies in oocytes have shown that N ramp2 can transport a number of divergent cations such as Fe$^{2+}$, Zn$^{2+}$, and Mn$^{2+}$ in a pH-dependent, electrogenic fashion associated with the symport of a single proton (12). In addition, transient overexpression of the wild type but not G185R N ramp2 in HEK293T cells results in a robust stimulation of cellular $^{55}$Fe uptake (15). Taken together, these results indicate that N ramp2 is the transferrin-independent system responsible for dietary iron absorption in the intestine. However, the ubiquitous expression of N ramp2 mRNA suggests that it may be involved in iron metabolism in other tissues as well. As opposed to N ramp1, where the cellular and subcellular localization of the protein have been established, the lack of isoform-specific, anti-N ramp2 antibodies has precluded the identification of the cell type and of the subcellular compartment expressing this protein. Such information is critical to elucidate the role of the N ramp2 protein in cellular iron metabolism. In particular, the demonstrated H$^+$-driven, Fe$^{2+}$ transport activity of N ramp2, as well as its expression in a wide variety of tissues, make it a likely candidate not only for transferrin-independent iron absorption in the intestine but also for the transferrin-independent uptake of iron in peripheral tissues. It is well established that acidification of the endosomal compartment causes Fe$^{3+}$ release from transferrin and that reductases then convert the Fe$^{3+}$ to Fe$^{2+}$, but the mechanism of transport of Fe$^{2+}$ across the endosomal membrane has not yet been elucidated.

Materials and Methods

Immunogens. For the production of isoform-specific polyclonal antisera directed against N ramp2, rabbits were immunized with fusion proteins containing glutathione S-transferase (GST) fused to a peptide segment derived from the amino terminal region of N ramp2 (residues 1–71; for amino acid numbering see reference 10). This peptide is in a region of the protein which is not conserved in other N ramp family members, including N ramp1 (18). The GST–N ramp2 fusion protein was constructed in the plasmid vector pGEX (Pharmacia) as follows. The N ramp2 sequence was amplified by PCR using oligonucleotides NF2 (5'-AAAGATCTATGGTGTTGATCC-3') and NR (5'-CCTGAAATTCGACCAGCAGTAGT-3') (nucleotides 1–268), and the full-length N ramp2 cDNA as template. The PCR product was digested with BglII and EcoRI and the fragment was subcloned into pGEX digested with BamHI and EcoRI to create the in-frame GST fusion protein. Overexpression of the N ramp2–GST fusion protein was carried out in large scale cultures of Escherichia coli and the protein was purified from bacterial lysates using glutathione–Sepharose 4B (Pharmacia) as previously described (19). Purified proteins were analyzed by 7.5% SDS-PAGE and excised from the gel after light staining with 0.05% Coomassie blue in ddH$_2$O.

Production of Anti-N ramp2 Antibodies. Polyclonal antibodies were produced in male New Zealand White rabbits as described previously (8). A system for affinity purification of the antibodies was devised using the same N ramp2 peptide fused to a second fusion partner, dihydrofolate reductase modified by the addition of eight consecutive histidine residues (his-DHFR). The fusion protein construct was made as described above, except that the PCR product was digested with EcoRI and the resulting overhangs repaired using the Klenow fragment of DNA polymerase I (Pharmacia) before digestion with BglII. The digested PCR product was ligated into BglII- and Smal-digested pQE40 plasmid vector (Qiagen). The in-frame his-DHFR–N ramp2 fusion protein construct was transformed into E. coli strain M15(phEp4) for expression (Qiagen). Purification was performed on Ni–NTA agarose according to experimental conditions suggested by the manufacturer (Qiagen). The polyclonal antisera directed against the GST fusion protein was purified against the his-DHFR fusion protein by a preparative immunoblot procedure (20). The anti-N ramp1 polyclonal antisera (8) was affinity purified against the corresponding N ramp1–GST fusion protein by the same protocol.

Cell Culture. The mouse monocyte-macrophage cell lines RAW 264.7 and J74a, the mouse Sertoli cell line TM 4, and the mouse kidney line mIMCD-3 were obtained from the American Type Culture Collection (ATCC). They were cultured in media and under conditions recommended by the ATCC. WEHI 3B (myelomonocyte), WEHI 231 (B lymphocyte), BL 141 (T lymphocyte), and 70Z/3 (pre-B cell) were cultured as described previously (21). Chinese hamster ovary (CHO) cells LR 73 (22) were grown in α-MEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 μg/ml penicillin, and 50 μg/ml streptomycin. All media and media supplements were purchased from Gibco BRL. Mouse macrophages were obtained by peritoneal lavage, as previously described (8). We have previously described the production and characterization of RAW macrophages expressing a transfected wild-type N ramp1 fused to a c-myc epitope (9). The c-myc-tagged N ramp2 expression plasmid was constructed by excising the c-myc-tagged N ramp2 cDNA from plasmid pBluescript (23) using SpeI and EcoR V sites from the polylinker, followed by cloning into the mammalian expression plasmid pCB6 (24). For expression in CHO cells, the same insert was cloned into the expression vector pMT2 (25). CHO cells were transfected by electroporation as described previously (9). Clones of stable transfectants were selected in geneticin (G418, 1 mg crude/ml final; Gibco BRL) for 10–14 d, picked and expanded individually, and tested for protein expression by immunofluorescence using the anti-c-myc tag monoclonal antibody 9E10 (Babco).

Immunoblotting and Immunoprecipitation. Crude membrane fractions from the various cells were prepared as described previously (27). Protein concentration of the membrane fraction was determined by the Bradford assay (BioRad). Proteins were separated on SDS-polyacrylamide gels and transferred by electroblotting to nitrocellulose membranes. For experiments where the membrane was to be stripped and reprobed, a polyvinylidene fluoride membrane was used (Westran; Schleicher and Schuell) to reduce protein loss from the membrane during stripping. Equal loading and transfer of proteins was verified by staining the blots with Ponceau S (Sigma Chemical Co.). The blots were blocked in T BST (10 mM Tris.Cl pH 8, 150 mM NaCl, 0.05% Tween 20, pH 8) plus 5% skim milk powder for 1 h at room temperature. Primary antibodies used were as follows: affinity purified rabbit anti-mouse N ramp2 (1:100 dilution); affinity purified rabbit anti-
mouse N ramp1 (1:200); mouse monoclonal anti c-myc-epitope tag 9E10 (Babco; 1:100), rat anti–mouse transferrin receptor (Biosource International; 1:200), and rat anti–mouse Lamp1 (1:200). Anti–rabbit, anti–rat, and anti–mouse secondary antibodies conjugated to horseradish peroxidase were used at 1:10,000 (Amer- sham). Chemiluminescence was used for detection of immune complexes on the immunoblot (ECL; Ams- ham). For immuno-precipitation, CHO cells and N ramp1 and N ramp2 CHO trans- fectants were metabolically labeled with [35S]methionine by incubating overnight in 100 μCi/ml of [35S]methionine (DuPont) in methionine-free DMEM (GIBCO BRL) containing 10% heat-inactivated, diazyl fetal bovine serum, 2 mM L-glutamine, and 2 mM HEPES. Immunoprecipitation was performed exactly as de- scribed previously (8).

Immunofluorescence Cells were grown on glass coverslips and fixed with 4% paraformaldehyde in PBS for 30 min at 4°C. Im-munofluorescence was performed as previously described (9) with the following modifications: incubation with the primary antibody was 1 h at 20°C for the anti-c-myc mouse monoclonal 9E10 (1:200; Babco), and the anti-Lamp1 rat monoclonal (1:200), or overnight at 4°C for the anti-N ramp2 antiserum (1:800) fol- lowed by anti–mouse, anti–rat, or anti–rabbit secondary antibodies conjugated to rhodamine (1:300) or FITC (1:200) (Jackson Immu-nochemicals). Immunofluorescence was analyzed with a N ikon microscope using the 100× oil immersion objective. Certain colocalization studies were carried out using a Zeiss laser confocal microscope with a 63× objective. Composites of confocal images were assembled and labeled using PhotoShop, M etamorph, and Freehand software. To label the lysosomal compartment, cells were incubated with 1 mg/ml lysine-fixable FITC–dextran (M ol-ecular Probes) in growth medium for 4 h at 37°C in 5% CO2. After washing, cells were incubated an additional 30 min to chase the dextran from the early endosomal to the lysosomal compart-ments. For identification of the early and recycling endosomal compartment, cells were incubated in serum-free medium con-taining 50 μg/ml FITC–transferrin (M olecular Probes) for 30 min at 37°C in 5% CO2. Phagosomes were formed by incubating the cells with 3 μm latex beads (Sigma Chemical Co.) diluted 1:200 in complete culture medium for 15 min at 37°C in 5% CO2. After treatments to identify the specific subcellular compartments, cells were fixed in 4% paraformaldehyde and processed for immu-nofluorescence.

G lysoside Treatment Endo-β-acetylglucosaminidase H (Endo H) and peptide N-glycosidase F (PNGase F) were obtained from N ew England Biolabs. All aliquots of membrane preparations from J774a and CHO cells were denatured before digestion in a buffer containing 0.5% SDS and 0.1 M β-mercaptoethanol for 2 min at 70°C. For Endo H digestion, samples were diluted twofold and incubated with 4,000 U of Endo H in 50 mM sodium citrate, pH 5.5, for 1 h at 37°C. The —Endo H controls were treated identi-cally except an equivalent volume of ddH2O was added in place of Endo H. For PNGase F digestion, samples were diluted twofold and incubated in 50 mM sodium phosphate, pH 7.5, 1% NP-40, with 500 U of PNGase F for 1 h at 37°C. The —PNGase F controls were treated identically except an equivalent volume of ddH2O was added in place of PNGase F.

R NA Isolation and Hybridization Studies Total cellular RNA was isolated using guanidinium–HCl solubilization and differ-ential ethanol precipitation (28). 20 μg of total cellular RNA were denatured in a formamide–formaldehyde mixture and loaded onto denaturing agarose gels containing 0.66 M formaldehyde. Blots were prehybridized in a solution containing 10% dextran sulfate, 1 M NaCl, 1% SDS, and heat-denatured salmon sperm DNA (200 μg/ml) at 65°C for 2–16 h. Hybridization was for 24 h at 65°C in the same buffer containing the radiolabeled probe (106 cpm/ml of hybridization buffer; specific activity 108 cpm/μg DNA). Blots were washed under conditions of increasing strin-gency up to 0.1× SSC and 0.1% SD5 at 65°C and then exposed to Kodak X R film with two intensifying screens at −70°C for 18 h to 7 d at −80°C.

Phagosome Fractionation Phagosomes were isolated from J774a cells by a modification of a method described previously (9). 10 subconfluent 150 mm dishes of each cell line were fed with a 1:200 dilution of blue-dyed latex beads (0.8 μm; Sigma Chemical Co.) in culture medium for 1 h at 37°C in 5% CO2. The cells were then washed in PBS and harvested in the presence of protease inhibitors (1 μg/ml leupeptin, 1 μg/ml aprotonin, 1 μg/ml pepstatin, and 100 μg/ml PM SF; all Boehringer M annheim) and recovered by centrifugation (2,000 g, 5 min). The cell pellets were washed and resuspended in homogenization buffer (8.5% sucrose, 3 mM imidazole, pH 7.4) and homogenized by passage through a 22G needle until 90% of the cells were broken, as monitored by light microscopy. Nuclei and unbroken cells were pelleted and the supernatant loaded onto a sucrose step gra-dient as follows: the supernatant was brought up to 40% sucrose by addition of 62% sucrose and loaded on top of a 1-ml 62% su-crose cushion. Layers of 2 ml of 35, 25 and finally 10% sucrose (all sucrose solutions wt/wt in 3 mM imidazole, pH 7.4, plus protease inhibitors) were sequentially added to the top of the tube, and the gradients were centrifuged at 100,000 g for 1 h at 4°C (SW 41; Beckman). Phagosomes were recovered from the 10–25% sucrose interface, washed with PBS containing protease inhibitors and recovered by a final centrifugation at 40,000 g in an SW 41 rotor at 4°C. The final pellets were resuspended in 2× Laemmlli sample buffer. Phagosomes prepared by this protocol have been previously shown to be free of endoplasmic reticulum (endoplasm, Bip, and calnexin) and Golgi apparatus (galactosyl transferase) contaminants (29).

Results To generate an isof orm-specific anti-N ramp2 antisera, a protein segment derived from the amino terminus of N ramp2 was selected based on its predicted antigenicity and its sequence divergence from N ramp1 (28% identity). A GST–N ramp2 fusion protein containing the amino terminal N ramp2 segment was produced and used for immu-nization, and the anti-N ramp2 fraction was further isolated from the immune serum by affinity purification against a second immobilized N ramp2–D H FR fusion partner. The antisera was tested for specificity by immunoblotting crude membrane fractions as well as by immunoprecipita-tion of [35S]methionine–labeled cell lysates from transfected CHO cell clones expressing either c-myc-tagged Nramp1 or c-myc-tagged N ramp2 proteins (Fig. 1). The anti-c-myc monoclonal antibody 9E10 (30) specifically recognized a species of apparent molecular mass 90–100 kD in the N ramp2-transfected CHO cells, and a protein of 85–95 kD in the N ramp1-transfected cells that were absent from extracts of untransfected CHO controls (Fig. 1, A and B, bottom). These protein species migrated as broad bands in SDS–acrylamide gels. In membrane preparations from N ramp2-transfected CHO cells (CHO N2), the affini-
most exclusively in mononuclear phagocytes, Nramp2 mRNA is expressed in most tissues (10–12). We questioned whether the two Nramp proteins would display an overlapping or mutually exclusive expression pattern. Northern blot analysis of total cellular RNA from a panel of murine hematological cell lines revealed a readily detectable level of Nramp2 mRNA expression in the macrophage lines RAW 264.7 and J774a as well as in Friend virus-transformed erythroleukemia (MEL) cells (Fig. 2A, top; exposure time 1 wk). A much lower level of expression of Nramp2 was found in other cell lines: WEHI 231 (B lymphocyte), WEHI 3B (melanoma), 70/2 (pre-B lymphocyte), and B1 141 (T lymphocyte). In comparison, Nramp1 mRNAs were restricted to the macrophage cell lines RAW 264.7 and J774a and is expressed at levels ~50-fold higher than that of Nramp2 (Fig. 2A, middle; exposure time 24 h). Thus, Nramp1 and Nramp2 mRNAs are coexpressed in macrophages.

To analyze Nramp2 protein expression in macrophages, membrane fractions were prepared from thiglycolate-induced primary mouse macrophages, from J774a and RAW 264.7 cultured macrophages. For comparison, membranes were also prepared from cell lines derived from tissues previously shown to express a high level of Nramp2 mRNA: the Sertoli cell line TM4 and the kidney inner medullary collecting duct line mIMCD-3 (17). Membranes were also prepared from control and Nramp2-transfected CHO cells, as well as from two cell lines expressing low levels of Nramp2 mRNA (Fig. 2B, WEHI 231 and WEHI 3B). The membranes were analyzed by immunoblotting with the anti-Nramp2 antibody (Fig. 2, B and C). The antibody detected a major heterogeneous immunoreactive protein species of broad electrophoretic mobility with an apparent molecular mass of 80–90 kD in all cells tested, with the exception of WEHI 231 cells and untransfected CHO cells. The protein was most abundant in TM4, RAW 264.7, J774a and MEL cells. The protein was also detected in the membranes prepared from primary mouse macrophages and mIMCD-3 cells, although at a lower level. WEHI 3B membranes showed the lowest level of Nramp2 expression.

Figure 1. After affinity purification, anti-Nramp1 and anti-Nramp2 antisera were tested for specificity and reactivity by immunoblotting against crude membrane fractions (A) as well as by immunoprecipitation of [35S]methionine-labeled cell lysates (B) from CHO cells or from the same cells transfected with a c-myc-tagged (tag sequence EQKLISEEDL) Nramp1 (CHON1) or a c-myc-tagged Nramp2 (CHON2). Protein extracts were separated by SDS-PAGE and either transferred to membranes (A) or exposed to x-ray films after immunoprecipitation (B). Antisera directed against Nramp2 (top), Nramp1 (center), or a commercially available mouse monoclonal anti-c-myc antibody (9E10; bottom) were used. The size of the molecular mass markers (in kD) is indicated to the right of the gels.

Figure 2. (A) Nramp2 mRNA expression in cultured cell lines. Northern blot analysis of total cellular RNA (20 μg) from mouse cell lines: BI 141 (T lymphocyte; lane 1), 70/2 (pre-B lymphocyte; lane 2), WEHI 231 (B lymphocyte; lane 3), WEHI 3B (melanoma; lane 4), RAW 264.7 (macrophage; lane 5), J774a (macrophage; lane 6), and MEL (erythroleukemia; lane 7). The RNA was electrophoresed in a denaturing agarose gel, followed by transfer to a nylon membrane and hybridization to gene-specific cDNA segments from either Nramp2 (top), Nramp1 (center), or glyceraldehyde phosphate dehydrogenase (G apdh, bottom). Exposure time was 7 d for Nramp2 and 1 d for Nramp1 and G apdh. (B) Nramp2 protein expression in cultured cell lines. Crude membrane fractions were prepared from different cultured cell lines as well as from thiglycolate-induced primary mouse macrophages (Mø) and were separated by SDS-PAGE. Immunoblotting was performed using the anti-Nramp2 antisera. (C) Glycosylation analysis of Nramp2 protein. Membrane fractions from J774a cells (lanes 1–4) and Nramp2-transfected CHO cells (lanes 5–7) were treated with endoglycosidases followed by electrophoresis and immunoblotting with the anti-Nramp2 antisera. Membrane fractions were either mock treated (lanes 1, 3, and 5) or incubated with Endo H (lane 2) or PNGaseF (lanes 4, 6, and 7). A longer exposure of lane 6 is shown in lane 7.
whereas WEHI 231 and untransfected CHO cells were negative for N ramp2 expression. In the positive membrane samples, the electrophoretic mobility and heterogeneity of the immunoreactive species varied, possibly due to different posttranslational modification of the protein in these cell types. Thus, N ramp2 is expressed in a wide variety of tissues, including macrophages, and macrophages coexpress Nramp1 and N ramp2.

The apparent mass of endogenous N ramp2 estimated by SDS-PAGE is considerably greater than the 62.3 kD molecular mass predicted by the primary amino acid sequence of the cDNA. Together with the broadness of the immunoreactive band, this anomalous mobility suggests that N ramp2 may be posttranslationally modified by glycosylation. To test this hypothesis, membrane fractions from J774a cells and N ramp2-transfected CHO cells were treated with endoglycosidases followed by electrophoresis and immunoblotting. N ramp2 was resistant to digestion with Endo H (Fig. 3 D), Similar results were obtained in parallel experiments using the c-myc-N ramp1 and c-myc-N ramp2-transfected RAW cells (data not shown), indicating that N ramp2 is not expressed in the lysosomal compartment. Thus, N ramp1 and N ramp2 clearly appear to have distinct, nonoverlapping subcellular sites of expression.

Since N ramp2 is implicated in cellular iron uptake, it appears logical that N ramp2 be present at the plasma membrane and/or in recycling endosomes. To label these compartments, CHO (Fig. 4) and RAW transfected cells (Fig. 5) were cultured in the presence of FITC–conjugated transferrin before fixation and immunostaining with the anti-c-myc antibody. Analysis by confocal microscopy indicated that, as expected, transferrin (green) stained both the plasma membrane (ring-like staining at the edge of the cells) and the recycling endosomes (subcellular punctate staining) (Fig. 4 B). A very similar and overlapping pattern was observed for N ramp2, as revealed by the anti-c-myc antibody (red, Fig. 4 A). Superimposition of the two images (Fig. 4 C) clearly identifies colocalization (yellow) of the two signals. Certain cells stained with FITC–transferrin but...
were negative for the c-myc staining, suggesting that although these cells are positive for the pSV2neo plasmid and are resistant to G418, they failed to express c-myc–tagged Nramp2. Such cells provide an internal control for the specificity of the anti-c-myc staining and for the overlapping staining with FITC–transferrin. Similarly, when untransfected CHO cells were identically processed and examined, the cells were not stained with the anti-c-myc antibody (Fig. 4 D), but displayed a normal pattern of staining with FITC–transferrin (Fig. 4 E). Finally, when the lysosomes of the Nramp2-transfected cells were stained with FITC–dextran (Fig. 4 H) and with the anti-c-myc antibody for Nramp2 (Fig. 4 G), no significant overlap between the two signals was detected (Fig. 4 I). A similar colocalization of Nramp2 and FITC–transferrin was noted in parallel experiments with RAW macrophages expressing c-myc–tagged Nramp2 (Fig. 5). These results confirm that Nramp1 and Nramp2 have nonoverlapping, subcellular localization, and that Nramp2 colocalizes with transferrin in the early recycling endosomal compartment.

To confirm the endosomal localization of Nramp2 determined in transfected CHO and RAW cells, we performed immunofluorescence in nontransfected cell lines that tested positive (MEL, TM4) or negative (WEHI 231) for Nramp2 expression by immunoblotting (Fig. 2). Immunofluorescence was performed with the anti-Nramp2 polyclonal antiserum and FITC–transferrin, and results are shown in Fig. 6. In MEL (Fig. 6, A and B) and TM4 (C and D) cells, the endogenous Nramp2 protein (B and D) showed extensive overlap with the distribution of FITC–transferrin (A + B = C) but not with lysosomal FITC–dextran (G + H = I). FITC–transferrin staining was observed in the untransfected CHO cells (E), but the cells were negative for anti-c-myc staining (D).

Figure 4. Colocalization of Nramp2 and transferrin in early endosomes was determined by double immunofluorescence and confocal microscopy in either CHO control cells (D–F) or CHO cells transfected with a c-myc–tagged Nramp2 protein (A–C and G–I). Cells were cultured in the presence of FITC–conjugated transferrin (A–F) or FITC–conjugated dextran (G–I) before fixation and immunostaining with the anti-c-myc tag antibody and a rhodamine-conjugated secondary antibody (A, D, and G). The slides were then examined by confocal microscopy, and the FITC (green; B, E, and H) and rhodamine (red; A, D, and G) images were overlayed (yellow identifies colocalization; C, F, and I). In the Nramp2-transfected CHO cells, the Nramp2 staining (A and G) showed extensive overlap with the distribution of FITC–transferrin (A + B = C) but not with lysosomal FITC–dextran (G + H = I). FITC–transferrin staining was observed in the untransfected CHO cells (E), but the cells were negative for anti-c-myc staining (D).
derived from the plasma membrane and are known to sequentially interact and acquire proteins from both early and late endosomes before their final fusion with lysosomes (29). We have shown that Nramp1 is acquired during the phagosomal maturation process, using the model system of latex bead–containing phagosomes (9). Latex bead–containing phagosomes are ideal for microscopic examination and can also be purified from cell homogenates by flotation on sucrose gradients. To determine whether Nramp2 can associate with the phagosomal membrane, J774a cells were fed latex beads for 1 h at 37°C, and the phagosomal fraction was isolated from cell homogenates by fractionation on sucrose gradient. Equal amounts of the purified phagosomal fraction and of a crude total membrane fraction were separated by SDS-PAGE, and the relative amount of endogenous Nramp2 in each sample was determined by immunoblotting. As shown in Fig. 7 A (left), Nramp2 was significantly enriched in purified phagosomes as compared to the crude membrane preparation. In these experiments, the Lamp1 protein (marker of the phagolysosome, center) and the transferrin receptor (marker of the plasma membrane, right) were used as controls. As expected, significant enrichment of Lamp1 was seen in the phagosomal fraction whereas the transferrin receptor was not enriched in phagosomes even though it is readily detectable in crude membrane fractions. These results suggest that Nramp2 becomes associated with the phagosome during its maturation to phagolysosome. Possible association of Nramp2 with latex beads phagosomes was further analyzed in J774a cells by immunofluorescence and confocal microscopy. J774a cells were fed latex beads and then fixed and processed by double immunofluorescence using anti-Nramp2 and anti-Lamp1 antibodies (Fig. 7 B). In J774a cells, the Nramp2 signal obtained with our antibody was weak, which limited the analysis. Nevertheless, in several of the sections analyzed a portion of the Nramp2 signal could clearly be seen...
In the current study, we have raised isoform specific anti-Nramp2 antisera and have used it to identify a number of structural and biochemical features of Nramp2 predicted from the primary amino acid sequence deduced from the cDNA. These analyses have shown that Nramp2 is an integral membrane protein which is extensively modified by N-linked glycosylation. As opposed to Nramp1, which is macrophage-specific, Nramp2 protein was found ubiquitously expressed in a majority of cell lines analyzed. The current study has also clearly established that Nramp2 and Nramp1 localize to distinct subcellular compartments. Whereas Nramp1 colocalizes with FITC-dextran in the lysosomal compartment, Nramp2 is not detectable in this compartment but rather shows clear colocalization with FITC-transferrin both at the plasma membrane and in recycling endosomes. The demonstration of Nramp2 expression in several peripheral tissues, the colocalization of Nramp2 and transferrin in plasma membrane and recycling endosomes, the iron transport properties of the Nramp2 protein, and the effect of Nramp2 mutations on iron metabolism in peripheral tissues are strong evidence that Nramp2 is responsible for transporting Fe^{2+} into the cytoplasm after acidification of the transferrin-positive endosome (Fig. 8). Interestingly, this acidification would simultaneously provide a gating mechanism for iron transport by Nramp2 and for the release of transferrin (Fig. 8). Indeed, the pH dependence of iron transport by Nramp2 has been demonstrated in several systems, including Xenopus oocytes (12), transfected HEK293T cells (14), and CHO cells (Gonovi, G., and P. Gros, unpublished results). Likewise, release of iron from transferrin and its subsequent release from endosomes is dependent on endosome acidification, which can be inhibited by bafilomycin (40) and concanamycin (41), specific inhibitors of the vacuolar H^+-ATPase, but is insensitive to the Na^+-K^+-ATPase inhibitor ouabain (41, 42). This suggests a critical role for the vacuolar H^+-ATPase in this process. Indeed, the association of vacuolar H^+-ATPase with transferrin-positive endosomal vesicles has been demonstrated by immunohistochemical means in LLC-porcine kidney epithelial 1 cells (43).
The demonstration that Nramp1 and Nramp2 are coexpressed in the same cell type with distinct subcellular localizations suggests possible functional parallels between Nramp1 and Nramp2 (Fig. 8). Both the yeast Smf1 and the mammalian Nramp2 proteins can transport Mn²⁺, with the latter also transporting Fe²⁺ and other divalent cations. Nramp2 and Smf1 share approximately 40% sequence identity within the conserved hydrophobic core (18). As the mammalian Nramp1 and Nramp2 proteins share almost 80% sequence identity within their hydrophobic cores, it is likely that Nramp1 is involved in the transport of divalent cations as well. The removal of such metabolically essential ions from the phagosomal space would provide an attractive explanation for the observed pleiotropic effect of Nramp1 mutations in vivo on the replicative potential of internalized microbes that inhabit the phagosomal space in macrophages. Additionally, the observation that a portion of Nramp2 associates with latex bead phagosomes in J774a cells suggests that Nramp2 may also play a role in depleting the phagosomal space of divalent cations necessary for microbial survival.

Despite considerable efforts, transport studies in CHO and RAW cells transfected and overexpressing Nramp1 protein have so far failed to demonstrate an Nramp1-mediated transport of either ⁵⁴Mn or ⁵⁵Fe transport in these cells (Govoni, G., and P. Gros, unpublished results). The distinct, nonoverlapping distribution of Nramp1 and Nramp2 reported here provides an explanation for this apparent lack of transport activity associated with Nramp1. While the two proteins may have the same transport potential, the observed targeting of Nramp2 to the lysosomal compartment would not cause a similar increased cellular uptake of a ligand presented in the extracellular milieu, although it could act on such ligand if present in the phagolysosomal space. Therefore, it is tempting to speculate that both Nramp1 and Nramp2 have similar transport function but act at different, nonoverlapping, intracellular sites (Fig. 8). If Nramp1 and Nramp2 do indeed transport the same substrates, it is also tempting to speculate that vesicular acidification via the vacuolar H⁺-ATPase may provide a key common gating mechanism for the activation of both transporters, through fusion with vacuolar H⁺-ATPase-positive vesicles (Fig. 8). Possible similarities and differences in the mechanism of action and regulation of Nramp1 and Nramp2 in macrophages are currently being investigated.

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