Human Natural Resistance-associated Macrophage Protein: cDNA Cloning, Chromosomal Mapping, Genomic Organization, and Tissue-specific Expression

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Summary

Natural resistance to infection with unrelated intracellular parasites such as Mycobacteria, Salmonella, and Leishmania is controlled in the mouse by a single gene on chromosome 1, designated Bcg, lty, or Lsh. A candidate gene for Bcg, designated natural resistance-associated macrophage protein (Nramp), has been isolated and shown to encode a novel macrophage-specific membrane protein, which is altered in susceptible animals. We have cloned and characterized cDNA clones corresponding to the human NRAMP gene. Nucleotide and predicted amino acid sequence analyses indicate that the human NRAMP polypeptide encodes a 550-amino acid residue membrane protein with 10–12 putative transmembrane domains, two N-linked glycosylation sites, and an evolutionary conserved consensus transport motif. Identification of genomic clones corresponding to human NRAMP indicates that the gene maps to chromosome 2q35 within a group of syntenic loci conserved with proximal mouse 1. The gene is composed of at least 15 exons, with several exons encoding discrete predicted structural domains of the protein. These studies have also identified an alternatively spliced exon encoded by an Alu element present within intron 4. Although this novel exon was found expressed in vivo, it would introduce a termination codon in the downstream exon V, resulting in a severely truncated protein. Northern blot analyses indicate that NRAMP mRNA expression is tightly controlled in a tissue-specific fashion, with the highest sites of expression being peripheral blood leukocytes, lungs, and spleen. Additional RNA expression studies in cultured cells identified the macrophage as a site of expression of human NRAMP and indicated that increased expression was correlated with an advanced state of differentiation of this lineage.

Tuberculosis remains one of the most important infectious diseases worldwide and is becoming even more threatening because of the recent emergence of multidrug-resistant and highly virulent strains of Mycobacterium tuberculosis (1). The mechanism of host defense against M. tuberculosis in general, and the mechanisms underlying long-term persistence and replication of tubercle bacilli inside mononuclear phagocytes in particular, remain unclear and need to be better understood. Epidemiological studies of tuberculosis have suggested that initial susceptibility to infection is under genetic control. In addition, racial differences in susceptibility to infection in genetically related and highly virulent strains of Mycobacterium tuberculosis (1). The mechanism of host defense against M. tuberculosis in general, and the mechanisms underlying long-term persistence and replication of tubercle bacilli inside mononuclear phagocytes in particular, remain unclear and need to be better understood. Epidemiological studies of tuberculosis have suggested that initial susceptibility to infection is under genetic control. In addition, racial differences in susceptibility to infection are under genetic control. In addition, racial differences in susceptibility to infection are under genetic control. In addition, racial differences in susceptibility to infection are under genetic control.
gene region on mouse chromosome 1, and have identified several candidate transcription units for Bcg (15). One of them was found to be expressed exclusively in reticuloendothelial organs and in mature tissue macrophages, the tissues and cell type known to phenotypically express the genetic difference at Bcg (9). This candidate gene, designated Nramp1 for natural resistance–associated macrophage protein, was found to encode a novel macrophage-specific polypeptide with predicted features characteristic of an integral membrane protein, including a minimum of 10 putative trans-membrane (TM) domains and two predicted N-linked glycosylation signals (15). The Nramp protein also contains a conserved consensus transport motif known as the “binding protein–dependent transport system inner membrane component signature” previously identified in a series of bacterial periplasmic transport proteins (16, 17). Nramp also shares similarity through this motif and other predicted structural aspects with the nitrate/nitrite concentrator of Aspergillus nidulans encoded by the emA gene (18), which raises the possibility that Nramp may be involved in the metabolism/transport of oxidation products of nitric oxide, a key effector of the cytotoxic arsenal of activated macrophages (19, 20). Finally, nucleotide sequence analyses of the Nramp cDNA showed that in 27 inbred mouse strains of either Bcg and Bcg supersusceptible phenotypes, the susceptibility trait was associated with a nonconservative glycine-to-aspartic acid amino acid substitution within predicted TM2 of the protein (21).

The goals of the present study were to (a) clone and characterize a cDNA for the human NRAMP homolog and analyze the pattern of conservation between the human and mouse proteins with respect to the predicted structural domains of the two proteins in general and the region bearing the mutation found in susceptible (Bcg) inbred mice in particular; (b) elucidate the genomic organization of the gene to obtain sequence information required for analyzing the role of human NRAMP in resistance to infection in humans; (c) investigate the expression pattern of the human gene in normal organs and cells and its potential involvement in macrophage-specific effector functions in relation to its proposed role in the metabolism of oxidized nitrogen intermediates.

Materials and Methods
cDNA Cloning and Nucleotide Sequencing. 5 × 105 independent clones from a human adult spleen cDNA library in λ gt10 vector (HL1039a; Clontech Laboratories Inc., Palo Alto, CA) were screened with an EcoRI-SacII fragment of the murine Nramp cDNA as hybridization probe (positions 1-1720 of published sequence (15)), under conditions of high stringency as described (22). The mouse cDNA probe was labeled to high specific activity by random priming (1-2 × 106 cpm/μg) (23). Filters were washed under conditions of increasing stringency up to 0.5 x SSC, 0.1% SDS at 60°C for 30 min. For nucleotide sequencing (24), single-stranded DNA templates were generated from pBluescript KS+/- subclones by superinfection with the M13 helper phage MK105. Double-stranded DNA plasmid templates were also used for nucleotide sequencing. Oligonucleotide primers used for sequencing were either derived from the plasmid vector or from the cDNA sequence itself. The complete cDNA sequence of human NRAMP cDNA was compiled from the analysis of two overlapping cDNA clones, designated H41 and H42. The nucleotide sequence data of the human NRAMP cDNA are available from EMBL/GenBank/DDBJ under accession number L32185.

Isolation of Genomic DNA Clones and Determination of the Exon/Intron Gene Structure. 5 × 105 independent clones from a human genomic DNA library constructed in the BamHI site of cosmid vector pWE15 (22) were screened for the presence of homologous human NRAMP sequences. Four independent but overlapping clones were purified, and their detailed restriction enzyme map was established for several enzymes. Oligonucleotide primers derived from either the plasmid vector, the human NRAMP cDNA, or genomic introns were used to determine the nucleotide sequence of genomic exons. Individual exon/intron boundaries were detected by a loss of identity between the genomic and cDNA nucleotide sequences and also by the presence of consensus donor and acceptor splicing signals at the point of divergence. The size of introns was determined by partial nucleotide sequencing, restriction mapping, and PCR amplification by use of exon-derived oligonucleotide primers.

Cell Culture. The human cell lines used were obtained from American Type Culture Collection (Rockville, MD) and included the acute myelogenous leukemia KG1 (CCL246), the promyelocytic leukemia HL60 (CCL240), the histiocytic lymphoma U937 (CRL1593), and the monocytic cell line THP1 (TIB202). Cells were cultured in Dulbecco's modification of Eagle medium with a high concentration of glucose (100 mM), 10% heat-inactivated FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO BRL, Gaithersburg, MD). Cultures were maintained in a humidified atmosphere consisting of 95% air and 5% CO2 at 37°C by passage of 1-2 × 106 cells/ml every other day. Cell viability was tested by trypan blue exclusion.

Amplification (RTPCR) of Human NRAMP cDNAs. Total or Poly(A) RNA was used as template and random hexamers as primers for the murine Moloney leukemia virus reverse transcriptase–mediated cDNA synthesis (GIBCO BRL), as previously described (25). Oligonucleotide primers HN1 (5'-CGTGGTGACAAGCCAAGGA-3', positions 417-435) and HN2 (5'-GACAAAAAGGTTTACTGATA-3', positions 988-970) were used to PCR amplify the alternatively spliced human NRAMP cDNAs. Two oligonucleotide primers of sequence 5'-ACCCACCGTGAAGGAGTGA-3' and 5'-GTTCCTACTTCTGCTTCCTGTA-3' were used for control amplification of a β2-microglobulin cDNA fragment (26). Reaction conditions for PCR amplification of human NRAMP cDNA were 94°C (1 min), 50°C (1.5 min), and 72°C (2 min) for 30 cycles. Reaction products were visualized by agarose gel electrophoresis after ethidium bromide staining and analyzed by Southern blot with a 300-bp human NRAMP cDNA fragment generated by PCR amplification (positions 515-819) as hybridization probe.

Computer-assisted Analyses. Analyses of nucleotide and predicted amino acid sequences were performed on the VAX/VMS server and computer of the Université de Montréal by use of the UWGCG software package (University of Wisconsin, Madison, WI) (27) and with the DNA Strider package (28). Homology searches in the National Center for Biotechnology Information data bases were performed by use of the BLAST server (National Library of Medicine, National Institutes of Health, Rockville, MD) (29). Identification and analysis of the Alu sequences present in the NRAMP gene was performed using Alu program (30) of the PYTHIA server.

Abbreviations used in this paper: NRAMP, natural resistance–associated macrophage protein; ORF, open reading frame; TM, trans-membrane.
**RNA Blotting Experiments.** Total RNA was extracted either from normal human spleen or cultured human cells as described (31). PMN from heparin-treated blood were purified by centrifugation on a density gradient (Histopaque™, Sigma Chemical Co., St. Louis, MO). The polyadenylated RNA fraction was selected by standard chromatography on oligo (dT)-cellulose (Pharmacia LKB Biotechnology, Piscataway, NJ) and analyzed by Northern blot after electrophoresis in formaldehyde-containing agarose gel (22). These membranes and human multiple tissues Northern filters I and II (Clontech) were prehybridized in a solution containing 10% dextran sulfate, 1 M NaCl, 1% SDS, and 200 μg/ml heat-denatured salmon sperm DNA for 2 h at 65°C. Hybridization for 16 h at 65°C was done in the same solution containing 2 × 10⁶ cpm/ml ²P-radiolabeled probe (human NRAMP PCR fragment, position 515–819; sp act 1–2 × 10⁹ cpm/µg DNA). Final wash conditions were 0.5 × SSC, 0.5% SDS at 65°C for 30 min.

**Results**

**Isolation of NRAMP cDNA Clones.** A human spleen cDNA library was screened under high stringency conditions with a mouse Nramp cDNA probe yielding five positive clones for a frequency of ~0.001%. Restriction enzyme mapping and nucleotide sequence analyses showed that these clones were overlapping and derived from the same mRNA species, although none of them were full length. The nucleotide sequence of the assembled cDNA and the predicted amino acid sequence of the encoded protein are shown in Fig. 1 A. The first in-frame initiator methionine codon was found at position 77 of the sequence and was followed by a segment of 1,647 nucleotides forming a single open reading frame (ORF) capable of encoding a protein of 550 amino acid residues with a calculated molecular mass of ~60 kD. A TGA termination codon was found in the same reading frame at position 20 upstream from the putative initiator ATG, indicating that the segment upstream position 77 was untranslated. A TAG termination codon located immediately downstream from glycine 550 (nucleotide position 1727) was followed by an additional 276 nucleotides of putative 3' untranslated sequence. No clear consensus polyadenylation signal (32) could be identified within this sequence.

**Analysis of the Predicted NRAMP Polypeptide.** Analysis of the predicted amino acid sequence of the human NRAMP polypeptide revealed that it was most likely an integral membrane protein. Highly hydrophobic amino acids such as leucine (84, 15%), isoleucine (34, 6%), valine (37, 7%), alanine (54, 10%), phenylalanine (32, 6%), and the apolar residue glycine (47, 9%) accounted for more than half (53%) of the polypeptide composition, whereas charged residues such as arginine/lysine (35, 6%) or aspartic/glutamic acid (34, 6%) accounted for only 12%. Hydrophathy analysis (Fig. 1 B) suggested that human NRAMP contained a minimum of 10 highly hydrophobic segments, which displayed minimal charge density. Although examination of the NH₂-terminal sequence of the protein did not reveal a classical cleavable signal sequence, this region contained a high proportion of hydrophobic or charged residues incompatible with membrane insertion but possibly acting as a hydrophobic cytoplasmic anchor. The human NRAMP protein showed within the segment separating putative TM domains 5 and 6, two predicted N-linked glycosylation sites at amino acid positions 224 (NXS) and 238 (NXT). The consensus sequence motif known as the "binding protein-dependent transport system inner membrane component signature" (17) was found in the segment separating predicted TM domains 6 and 7 (positions 373–392, dotted underline in Fig. 1 A). This motif, which has been evolutionarily conserved from prokaryotes to eukaryotes, is predicted to be localized in cytoplasmic loops of periplasmic transport proteins and possibly mediates interaction of the hydrophobic membrane anchors with peripheral ATP binding subunits to energize these transporters (16). The combined analysis of the hydrophathy profile and the location of known structural/functional motifs suggested the following topology for human NRAMP. The NH₂ terminus would be cytoplasmic and followed by 10 consecutive TM domains forming five membrane loops, placing the COOH terminus on the cytoplasmic side. This arrangement would position the TM 5–6 loop as extracytoplasmic (predicted sites for N-linked glycosylation), whereas the TM 6–7 loop carrying the consensus transport motif would be intracytoplasmic.

**Homology Between Mouse and Human NRAMP Proteins.** Comparison of the human and mouse predicted NRAMP protein sequences revealed a remarkable degree of conservation between the two polypeptides, with 88% identical residues and 93% overall sequence similarity. Substitutions were not randomly distributed along the length of the protein but were clustered within discrete predicted structural domains (Fig. 2, E and F). The extreme NH₂ (residues 1–50, 66% identity) and COOH-terminal ends (residues 517–550, 74% identity) of the two proteins were the least conserved segments, and gaps of three residues (positions 20–22 of mouse sequence) and one residue (position 546 of human sequence) had to be introduced to optimize alignment in these segments. In addition, the predicted second (TM 3–4 segment, positions 218–240; 63% identity) and third extracytoplasmic loops (TM 5–6 segment, positions 310–349; 67% identity) showed significant sequence divergence. The fifth putative intracytoplasmic loop located between TM 8–9 (positions 450–468; 61% identity) and the last predicted TM 10 domain (positions 496–517; 67% identity) also contained a cluster of substitutions that were mainly conservative. The rest of the NH₂-terminal third up to and including TM 3 (residues 50–217) and the segment overlapping TM 6 to TM 8 (residues 340–462) were the most conserved portions (97% identical, 99% similar; Fig. 2, E and F). The two predicted phosphorylation sites by protein kinase C identified in the mouse sequence (15) were not conserved in the human protein; instead, a new one was found within the NH₂-terminal region (pst 54, Fig. 1 A). By contrast, both proteins have retained the two predicted N-linked glycosylation sites within the third predicted extracellular loop (TM 5–6 interval). As expected, the binding protein dependent transport system inner membrane component signature was highly conserved in human NRAMP, including a conservative arginine-to-lysine substitution at last position, 392, of the motif. The highly hydrophobic predicted membrane-associated domains of the proteins were strikingly conserved in the two species. Predicted
Figure 2. Genomic organization of the human NRAMP gene region. (A) Physical map of the NRAMP gene region on human chromosome 2q delineated by the TNP-1 (2q35-36, proximal) and VIL (2q35, distal) genes. The maximal distance separating NRAMP and VIL is estimated at 220 kb. (B) Position and relationship of cosmid clones hybridizing to the NRAMP cDNA and used to characterize the gene. (C) The position of the recognition sites of restriction enzymes NotI (N), KpnI (K), EcoRI (E), and BamHI (B) within the cosmid clones are indicated. (D) Genomic organization and exon/intron structure of the human NRAMP gene. Exons are indicated by filled boxes numbered with roman numbers, whereas intervening and flanking sequences are represented by a thin line. The empty box between exons IV and V corresponds to the alternatively spliced Alu-derived exon (see text). The direction of transcription is from left to right. (E) Schematic representation of the human NRAMP mRNA and the putative encoded product. The thin lines represents the 5' and 3' untranslated sequences, and the boxed region indicates the mRNA coding region. The boxes numbered 1-14 identify intervening sequences in the precursor mRNA. The corresponding scales for the gene (1 kb) and the encoded polypeptide chain (10 amino acids) are indicated. (F) Schematic representation of the nonidentical amino acid residues at homologous positions of the mouse and human NRAMP polypeptide chains. Conservative substitutions are represented by short single bars, whereas nonconservative substitutions are shown as long double bars.

TM 1-8 were identical in sequence (except for a single isoleucine-to-valine conservative substitution in TM 6), including four charged amino acids in TM 1-3 and 7. This suggests that these TM domains and residues play key structural and/or functional roles in this protein.

Cloning of the Human NRAMP Gene. Genetic linkage and physical mapping studies have established that the mouse Bcg/Nramp gene maps on the proximal portion of chromosome 1, very close (125 kb) to the intestinal protein gene villin (14). This chromosomal region forms part of a large syntenic...
Table 1. Splice Junctions in the NRAMP Gene

| Exon/intron number | Exon length bp | Exon 3' junction | Intron 5' junction | Intron length bp | Intron 3' junction | Exon 5' junction | Intron type |
|--------------------|----------------|------------------|--------------------|------------------|--------------------|------------------|-------------|
| I                  | >73            | ATG ACA G         | gtagttagtggc       | 585              | gggccccccacag      | GT GAC AAG       | 1           |
| II                 | 143            | ACA AAA CCG      | gtgggatgtgg        | ~1,100           | aacccccaaacag      | GGC ACC TTC     | 0           |
| III                | 123            | GGA TTC AAA      | gtaactaagtgc       | ~800             | gcctccctcag        | CTT CTC TGG     | 0           |
| IV                 | 120            | TAC CCT AAG      | gtagctttggg        | ~1,200           | tctcccccatag       | GTG CCC CGC     | 0           |
| V                  | 107            | GCT GGA CG       | gtacccaccccaag     | ~450             | ctcctgtttcag       | A ATC CCA      | 2           |
| VI                 | 71             | AAC TAC G        | gtgggtgcacac       | 326              | tctgccttcag        | GG CTG CGG      | 1           |
| VII                | 68             | 715              | GGC TAT GAG        | ~300             | tctccctgcag        | TAT GTG GTG     | 0           |
| VIII               | 156            | CTG GTC AAG      | gtgccagaggg        | ~1,500           | tgtaccttcgtag      | TCT CGA GAG     | 0           |
| IX                 | 159            | CAG GCT GCG      | gtgagacacact       | ~1,200           | ggtaccccaacag      | TTC AAC ATC     | 0           |
| X                  | 90             | 1,120            | TAC CAG GGG        | 87               | ctcctgttcag        | GGC GTG ATC     | 0           |
| XI                 | 120            | GTG ATG GAG      | gtagggagggg        | ~1,500           | cccccccacccag      | GTG ACC CTG     | 0           |
| XII                | 150            | AGC CTG CTG      | gtgagatagcc        | ~900             | tgtctctccccag      | CTC CGG TTC     | 0           |
| XIII               | 74             | 1,464            | AAT GGC CT         | ~400             | cctgctcccccag      | CTT GAC AGC     | 2           |
| XIV                | 154            | 1,618            | ACC TAC CTG        | 139              | cccccccccccag      | GTC TGG ACC     | 0           |
| XV                 | >389           | 2,007            |                    |                  |                    |                  |             |

Consensus

Sequences: N G / G T R V G / G Y Y Y Y Y Y N Y A G / N

Frequency (%): 100 85.7 100 100 100 78.6 85 71.5 92.8 85.7 100 100 100 100 100 100 100

* Positions in the cDNA sequence.

† Length of the introns was determined by a combination of PCR and restriction enzyme mapping of genomic plasmid subclones.

§ Intron type is indicated by 0, 1, and 2 when a splice junction occurs between codons, after the first nucleotide of the codon, and after the second nucleotide of the codon, respectively.

N = A/C/G/T, IL = A/G, Y = C/T/, V = A/G/C.

donor and acceptor sites conformed to the AG/GT rule and gave a consensus splicing sequence for the gene, which was strictly in accordance with the consensus proposed by Mount (35). Although exon 1 was defined as containing the first nucleotide of the cDNA sequence, the transcription initiation site of the gene has not yet been accurately mapped.
of high stringency. NRAMP mRNA expression was found to be highly restricted to certain tissues; in these tissues, two major hybridizing mRNA species of ~2 and 4 kb in size were coexpressed. The size of the shorter species at 2 kb was consistent with the 2,007-bp size of the reconstructed NRAMP cDNA (Fig. 1 A), suggesting that the cDNA reported here is near or full length. The origin of the larger 4-kb mRNA remains unclear, but could result from the use of alternative polyadenylation signals in the gene. The site of highest NRAMP mRNA expression was found to be PBL. This very high expression was not unique to the individual used to produce the blot, because a comparable signal was obtained with RNA from eight additional and unrelated healthy volunteers (data not shown). A high expression level was observed in lungs and a moderate to low level detected in spleen and liver, respectively. Normalization of hybridization signals with actin (Fig. 4 B) and GAPDH (data not shown) control probes revealed that NRAMP expression in spleen was much higher than in liver. The high levels of NRAMP expression detected in PBL, lung, and spleen, together with the absence of expression in other tissues, suggested that expression of this gene may be restricted to cells of the reticuloendothelial system such as the macrophage/monoocyte lineage. That possibility was further investigated in vitro by use of RT-PCR analysis of RNA from a series of human cell lines showing characteristics of this lineage. Cell lines KG-1, HL 60, U 937, and THP1 represent immature precursors of the myelomonocytic lineage blocked by immortalization/transformation in a proliferative state at different stages of differentiation towards phagocytic leukocytes (40). KG-1 cells are the closest to normal bone marrow precursors, whereas THP1 are closest to the monocytic cell type. Results shown in Fig. 4 C indicate that all cell types analyzed expressed NRAMP mRNA. Interestingly, an apparent correlation between the level of expression and the commitment of the cell towards the phagocytic type was noted.

Discussion

Southern blot analysis of total human genomic DNA with the mouse Nramp cDNA probe under low stringency conditions identified two sets of hybridizing fragments, a set of strongly hybridizing ones and a set of a weakly cross-hybridizing ones, suggesting the presence of at least two sequence-related NRAMP genes in humans (Vidal, S., and P. Gros, unpublished observations). Therefore, high stringency conditions were used to isolate a human NRAMP homolog. Sequence analysis of the positive clones identified a very high degree of nucleotide sequence conservation with the mouse counterpart (Fig. 1 A), and mapping studies indicated that the corresponding human gene mapped on human 2q35 proximal to the VII gene on a chromosomal segment homologous to the portion of mouse 1 carrying Bcg/Nramp (Fig. 2 A) (33). Additional mapping studies excluded human chromosome 2 as the site for the second gene identified by cross-hybridization in Southern blotting (data not shown). Together, these results indicated that the cDNA and gene
capital letters in the nucleotide sequence, and the encoded amino acid sequences are indicated below. Alternative splicing of the Alu-encoded exon of a portion of the

include alternatively spliced exons from other human genes sharing similarity with the predicted sequence encoded by the alternatively spliced Alu-encoded exon. These

87% identical positions, indicated by stars. Analysis of the divergent positions pattern indicates that this Alu belongs to the Sx family (36). (C) Polypeptide strand of the inverted Alu sequence found in intron 4 of the

Introduces a frameshift resulting in the appearance of a termination codon in the following exon V. (B) Nucleotide sequence alignment of the sense strand of the consensus Alu sequence with the sense strand of the consensus Alu sequence (upper line) gave

51 nucleotides upstream of the proposed murine ATG, and then disappeared abruptly. Translation of this 51-nucleotide

identified here correspond to the human homolog of mouse Nramp. Comparison of the nucleotide and predicted amino acid sequences of the mouse and human NRAMP cDNAs revealed one important difference at the 5' end of the mRNA. In the previously reported mouse Nramp cDNA sequence (15), the first in-frame initiator methionine was followed by an ORF of 1,452 nucleotides, as opposed to the 1,647 nucleotides ORF detected in human NRAMP. At the 5' end, nucleotide sequence conservation between the two species extended 51 nucleotides upstream of the proposed murine ATG, and then disappeared abruptly. Translation of this 51-nucleotide putative 5' UT segment showed that the corresponding amino acid sequence would be identical to its human counterpart (Fig. 1A). In addition, the abrupt loss of nucleotide sequence similarity between the human and mouse sequences in this region coincided with the position of intron 2 in the human gene (Table 1). Subsequent S1 mapping experiments and genomic DNA sequencing verified that the 5' end of the published mouse Nramp cDNA sequence contains an unspliced intron. Additional mouse Nramp cDNA clones were therefore constructed, and their analysis revealed an additional 64 amino acid residues upstream of the previously suggested ATG (Govoni, G., and P. Gros, unpublished results; sequence data available from EMBL/GenBank/DDBJ under accession number L36019).

Figure 3. Identification of an alternatively spliced exon encoded by an inverted Alu Sx sequence in intron 4 of the NRAMP. (A) Nucleotide sequence of a portion of the NRAMP gene encompassing part of intron 3, exon IV (positions 71-190), parts of intron 4, exon V (1373-1379), and part of intron 5. The inverted Alu sequence identified within intron 4 is underlined. Exons (including the alternatively spliced exon within intron 4) are in bold capital letters, and the encoded amino acid sequences are indicated below. Alternative splicing of the Alu-encoded exon introduces a frameshift resulting in the appearance of a termination codon in the following exon V. (B) Nucleotide sequence alignment of the sense strand of the consensus Alu sequence with the sense strand of the consensus Alu sequence (upper line) gave 87% identical positions, indicated by stars. Analysis of the divergent positions pattern indicates that this Alu belongs to the Sx family (36). (C) Polypeptide sequences from other human genes sharing similarity with the predicted sequence encoded by the alternatively spliced Alu-encoded exon. These include alternatively spliced exons (alt. spl.) from the DAF gene (complement decay accelerating factor; 37), the B1x-INT gene (beta-integrin; 38) and a sequence from the XEH. 8c-expressed sequence derived from a candidate gene for X-linked retinopathy (39). Only nonidentical residues are shown. Numbers in italics above each polypeptide sequence indicate the type of intervening sequences and position of the splice site for each particular Alu-encoded sequence. These sequence data are available from EMBL/GenBank/DDBJ under accession number L36019.
be 20, 12, and 2, respectively, resulting in an almost perfect
distribution of charged residues with respect to the
membrane. For positively charged residues
would be intracytoplasmic, 12 would be extracytoplasmic,
and 2 would map within predicted TM domains; for nega-
tively charged residues, the corresponding distribution would
be 20, 12, and 2, respectively, resulting in an almost perfect
charge balance on either side and also within the membrane
(net charge of +1). Careful scrutiny of the hydropathy profile
(42) and hydrophobic moment analysis (43) of the sequence
for the presence of hydrophobic or amphipathic α helices
identified two additional putative TM domains in the NH2-
terminal segment of NRAMP. The first one would be
delineated by residues 58–78 between the initiator ATG and
the identified TM 1, whereas the second one would map at
positions 137–158, between the identified TM 1 and TM 2.
In addition, these putative additional TM domains map in
a region of the molecule that is highly conserved in the mouse
homolog. However, the latter additional TM domain would
be thermodynamically disfavored, requiring the stable inser-
tion of three negatively charged residues in the membrane.
Therefore, NRAMP would encode a minimum of 10 and
a maximum of 12 putative TM domains.

Sequence comparison of the human and mouse proteins
revealed a remarkably high degree of conservation between
the two species (88% identity, 93% similarity). Whereas con-
servation of the predicted membrane topology, N-linked
glycosylation signals, and “transport motif” was expected,
the sequences of 10 out of the 12 possible TM domains were
precisely conserved, including the presence of several charged
residues in these domains. This extremely high degree of con-
servation is somewhat surprising since conservative substitu-
tion of hydrophobic amino acids are usually tolerated in TM
domains without alteration or loss of function between
homologs of the same proteins (44). This is of particular in-
terest since the only amino acid sequence variation detected
in Nrramp between inbred mouse strains of opposite geno-
types at Bcg is a single nonconservative glycine-to-aspartic
acid substitution within predicted TM 2 identified in innately
susceptible (Bcg*) strains (15, 21). The observation that this
TM domain in general and glycine in particular are precisely
conserved in human and mouse (this study) but also in the
homologous region of the rat and chicken gene (21) strongly
argues that this domain is important for function and that
nonconservative substitutions in this region are likely to affect
the function of this putative transporter.

Although the putative NRAMP substrate or mechanism
of transport remain to be identified, we have detected in
molecular database searches a few sequences locally similar
to NRAMP, in particular, in the region of the consensus trans-
port motif. These short “expressed sequence tags” (45) are
issued from plant genome research projects. The rice (O. sativa;
accession number L13732). This novel additional sequence was
homologous to that predicted for human NRAMP and was
used in our analysis (Figs. 1 A and 2 F). During the course
of these studies, portions of the 5’ end sequence of the mouse
cDNA and exon 2 of the human gene were independently
reported (41). These partial sequences were found identical
to those reported here.

Analysis of the full-length predicted amino acid sequence
of the NRAMP polypeptide, including the presence of land-
mark functional and structural features, has led us to pro-
pose a possible topology in which the NH2- and COOH-
terminal segments of the protein would be intracytoplasmic
and separated by 10 putative TM domains. The predicted
10-TM domain configuration has interesting consequences
on the distribution of charged residues with respect to the
membrane. For positively charged residues (Arg/Lys), 21
would be intracytoplasmic, 12 would be extracytoplasmic,
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port motif. These short “expressed sequence tags” (45) are
issued from plant genome research projects. The rice (O. sativa;
accession number L13732). This novel additional sequence was
homologous to that predicted for human NRAMP and was
used in our analysis (Figs. 1 A and 2 F). During the course
of these studies, portions of the 5’ end sequence of the mouse
cDNA and exon 2 of the human gene were independently
reported (41). These partial sequences were found identical
to those reported here.

Analysis of the full-length predicted amino acid sequence
of the NRAMP polypeptide, including the presence of land-
mark functional and structural features, has led us to pro-
pose a possible topology in which the NH2- and COOH-
terminal segments of the protein would be intracytoplasmic
and separated by 10 putative TM domains. The predicted
10-TM domain configuration has interesting consequences
on the distribution of charged residues with respect to the
membrane. For positively charged residues (Arg/Lys), 21
would be intracytoplasmic, 12 would be extracytoplasmic,
and 2 would map within predicted TM domains; for nega-
tively charged residues, the corresponding distribution would
be 20, 12, and 2, respectively, resulting in an almost perfect
charge balance on either side and also within the membrane
(net charge of +1). Careful scrutiny of the hydropathy profile
(42) and hydrophobic moment analysis (43) of the sequence
for the presence of hydrophobic or amphipathic α helices
identified two additional putative TM domains in the NH2-
terminal segment of NRAMP. The first one would be
delineated by residues 58–78 between the initiator ATG and
the identified TM 1, whereas the second one would map at
positions 137–158, between the identified TM 1 and TM 2.
In addition, these putative additional TM domains map in
a region of the molecule that is highly conserved in the mouse
homolog. However, the latter additional TM domain would
be thermodynamically disfavored, requiring the stable inser-
tion of three negatively charged residues in the membrane.
Therefore, NRAMP would encode a minimum of 10 and
a maximum of 12 putative TM domains.

Sequence comparison of the human and mouse proteins
revealed a remarkably high degree of conservation between
the two species (88% identity, 93% similarity). Whereas con-
servation of the predicted membrane topology, N-linked
glycosylation signals, and “transport motif” was expected,
the sequences of 10 out of the 12 possible TM domains were
precisely conserved, including the presence of several charged
residues in these domains. This extremely high degree of con-
servation is somewhat surprising since conservative substitu-
tion of hydrophobic amino acids are usually tolerated in TM
domains without alteration or loss of function between
homologs of the same proteins (44). This is of particular in-
terest since the only amino acid sequence variation detected
in Nrramp between inbred mouse strains of opposite geno-
types at Bcg is a single nonconservative glycine-to-aspartic
acid substitution within predicted TM 2 identified in innately
susceptible (Bcg*) strains (15, 21). The observation that this
TM domain in general and glycine in particular are precisely
conserved in human and mouse (this study) but also in the
homologous region of the rat and chicken gene (21) strongly
argues that this domain is important for function and that
nonconservative substitutions in this region are likely to affect
the function of this putative transporter.

Although the putative NRAMP substrate or mechanism
of transport remain to be identified, we have detected in
molecular database searches a few sequences locally similar
to NRAMP, in particular, in the region of the consensus trans-
port motif. These short “expressed sequence tags” (45) are
issued from plant genome research projects. The rice (O. sativa;
accession number D15268) and Arabidopsis (A. thaliana;
EMBL/GenBank/DDJB accession number Z30530) protein
sequences exhibited 46% identity (79% similarity) and 51%
identity (73% similarity), respectively (Cellier, M., and P.
Gros, unpublished results). Given the very low probability
for these TBLASTN hits to occur by chance (p = 5.0 e-17
and p = 1.7 e-09, respectively), these similarities in primary
amino acid sequences are likely to reflect structural and/or
functional homology between NRAMP and the corre-
sponding plant proteins (29). The intriguing link between
NRAMP and these plant proteins is under investigation with
respect to the “nitrate transporter” hypothesis for NRAMP function (15).

The juxtaposition of intron positions on the primary amino acid and predicted structural features of the NRAMP polypeptide revealed that the genomic organization of NRAMP had a highly modular structure. This is in agreement with previous observations suggesting that introns often mark turns or edges in protein secondary structure, including integral membrane proteins (46). Characterization of the intron/exon structure of the NRAMP gene revealed the presence of an unusual alternatively spliced exon contained within the normal intron 4. Nucleotide sequence analysis of intron 4 revealed that this new exon was defined by consensus-splicing signals mapping within the boundaries of an Alu element inserted in a reverse orientation with respect to gene transcription. This Alu element was found to belong to the ancient Sx family of Alu sequences (50% of common Alu) (36). The type of splicing event detected here and restricted to Alu elements inserted in the orientation opposite to transcription has been proposed to be an evolutionarily important mechanism for the generation of protein sequence diversity (49). Such insertion/splicing events have been shown to involve Alu elements belonging to the Sx and J subfamilies, which inserted in the genome at least 40 millions years ago (48), and cryptic splice sites could have been activated within the Alu element as a result of mutations subsequent to insertion (47). Although both alternatively spliced forms were expressed in vivo (Fig. 4 C), the physiological consequences of the alternatively spliced mRNA variant remain unclear as addition of the novel exon would result in a severely truncated protein. The predicted polypeptide encoded by the novel exon in NRAMP was similar to several other polypeptides surveyed (Fig. 3 C). Interestingly, in the case of the human complement decay accelerating factor (37) and integrin β1s genes (38), Alu-mediated alternative splicing events such as that observed here have been shown to give rise to distinct protein isoforms with different subcellular localization and biological activities.

Northern blot analysis revealed NRAMP expression in reticulo-endothelial organs and suggested that, by analogy with the mouse, the macrophage would be an important site of NRAMP mRNA expression in humans. In support of this proposal was the observation that of four committed progenitors of the monocytic lineage blocked at different stages of differentiation, all expressed NRAMP (Fig. 4 C). On the other hand, the relative abundance of NRAMP mRNA in PBL compared with spleen could not be explained solely on the basis of different numbers of the same cell type (either mature macrophages or other cell type of the monocytic lineage) present at these two sites. In addition, the high levels of expression detected in human lungs is in sharp contrast to the absence of expression of the mouse homolog in the same tissue (15). Several possibilities can be put forward to explain these findings. The first is that macrophages at different stages of differentiation or activation may be present in different amounts at these sites and may themselves express widely different amounts of specific mRNA (49). This is in agreement with our finding that NRAMP expression is increased concomitantly with the state of differentiation of the model cell types used in our analysis of the monocytic pathway (Fig. 4 C). The second possibility is that more than one cell type expresses NRAMP, and that cells of the granulocytic and/or lymphocytic pathways may also express the gene. The absence of NRAMP expression in adult thymus (Fig. 4 A) argues against a strong level of expression in lymphocytes. The PBL isolation procedure used here depleted most of granulocytes; however we cannot exclude the possibility that the remaining fraction of these phagocytic cells contributed to the signal detected in our analysis. A third alternative is that the human lung RNA sample analyzed here may have originated from a patient with an unusually high proportion of monocytes or other NRAMP-positive cells brought about by specific infection, inflammation, or other conditions. Such cells may not be present in normal lung tissue from mice raised in captivity under semisterile conditions. Finally, the levels of the mouse and human NRAMP mRNAs may be regulated very differently at the transcriptional and posttranscriptional levels during maturation and/or activation of the macrophage in the two species. This situation is reminiscent of the gene encoding the inducible nitric oxide synthase expression in the macrophage, where strikingly different mechanisms of induction have been proposed for the two genes (50–52). The cloning of genomic sequences reported here for human NRAMP, and the identification of cell lines showing differentiation-dependent expression, should help define the cis-acting sequences and trans-acting cellular factors controlling NRAMP gene expression.

The identification of a human NRAMP homolog, its chromosomal localization, the characterization of the nucleotide sequence of its cDNA, and the elucidation of the exon/intron structure of the gene reported here, together provide the necessary tools to test the possible role of this gene in differential resistance/susceptibility of humans to infection with intracellular pathogens such as Mycobacteria, Salmonella, and Leishmania. This proposal can now be formally tested by segregation analysis or direct DNA sequencing of the NRAMP gene from large informative familial pedigrees from endemic areas of such diseases.

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