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Nramp defines a family of membrane proteins

(gene family/protein structure/topology prediction)

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ABSTRACT **Nramp (natural resistance-associated macrophage protein) is a newly identified family of integral membrane proteins whose biochemical function is unknown. We report on the identification of Nramp homologs from the fly *Drosophila melanogaster*, the plant *Oryza sativa*, and the yeast *Saccharomyces cerevisiae*. Optimal alignment of protein sequences required insertion of very few gaps and revealed remarkable sequence identity of 28% (yeast), 40% (plant), and 55% (fly) with the mammalian proteins (46%, 58%, and 73% similarity), as well as a common predicted transmembrane topology. This family is defined by a highly conserved hydrophobic core encoding 10 transmembrane segments. Other features of this hydrophobic core include several invariant charged residues, helical periodicity of sequence conservation suggesting conserved and nonconserved faces for several transmembrane helices, a consensus transport signature on the intracytoplasmic face of the membrane, and structural determinants previously described in ion channels. These characteristics suggest that the Nramp polypeptides form part of a group of transporters or channels that act on as yet unidentified substrates.**

In mice, natural resistance to infection with unrelated intracellular parasites is controlled by the *Bcg* locus, which modulates the cytostatic/cytocidal activity of professional phagocytes (1, 2). By positional cloning a candidate gene (*Nramp1*) for *Bcg* has been isolated (3). *Nramp1* is expressed exclusively in macrophages and polymorphonuclear leukocytes and encodes a polypeptide with features typical of integral membrane proteins (3, 4), including 10–12 possible transmembrane (TM) domains, a glycosylated extracytoplasmic loop, and an intracytoplasmic consensus transport signature (5). This signature was originally identified in the integral membrane components of bacterial traffic ATPases (6) and subsequently found in a few eukaryotic membrane proteins, including a nitrate/nitrite concentrator of *Aspergilus nidulans* (7). This led to speculation that the Nramp1 protein may be involved in the metabolism or transport of nitrite and nitrate ions, two oxidation products of nitric oxide, a key microbicidal molecule produced by activated macrophages (3). Sequence analysis of Nramp1 in mouse strains resistant and susceptible to infection (8) and gene targeting experiments (9) have shown that a single $Gly^{169} \rightarrow$ Asp substitution in TM domain 4 abrogates Nramp1 function.

Nramp1 is part of a small gene family of at least two and probably three members in mouse and humans, and we have recently characterized a second *Nramp* gene, *Nramp2* (10–12). This gene maps on mouse chromosome 15 (*NRAMP2*, human chromosome 12q13) and, as opposed to the restricted expression of *Nramp1*, is expressed in all tissues tested. The mouse Nramp1 and Nramp2 proteins share 66% identity (77% similarity) and show nearly identical hydropathy profiles, resulting in indistinguishable predicted secondary structures (11, 12). The sequence conservation between Nramp1 and Nramp2 (and also among Nramp1 bovine and avian homologs) is particularly striking within the TM domains (86% similarity), suggesting that these domains play a particularly important structural and/or functional role common to these proteins. However, the biochemical function of Nramp1 and Nramp2 remains unknown.

We have used either direct cDNA cloning (plant), analysis of the malvolio fly mutant (13), or computer-assisted searches of databases (yeast) to identify *Nramp* homologs from distant eukaryotes, which together form a family of membrane transporters.

METHODS

Sequence alignments were produced with the programs PILEUP (ref. 14; Genetics Computer Group) and MACAW (15). Protein secondary structure predictions were performed with peptide structure calculations (Genetics Computer Group), hydropathy profiling (16), hydrophobic-moment calculations (17), TM helix prediction based on multiple sequence alignment of related proteins (18), and TM topology prediction (MEMSAT; ref. 19). Periodicity of conserved/variable residues in multiple sequence alignments was determined by a Fourier transform procedure (20), and substitution tables for lipid-facing residues (21) were used to identify a putative lipid-accessible face of the helices.

RESULTS AND DISCUSSION

Identification and Primary Amino Acid Sequence Analysis of Nramp-Related Sequences. Through routine searches of the National Center for Biotechnology Information databases with the BLAST server, three expressed sequence tags from the plants *Oryza sativa* (D15268, D25033) and *Arabidopsis thaliana Oryza* (Z30530) were identified that share considerable identity with Nramp1 (57%, 46%, and 48%, respectively; data not shown). A full-length cDNA clone corresponding to D15268 was isolated (*OsNramp1*) and found to encode a protein similar to mammalian Nramp. The *Drosophila melanogaster* mutant malvolio shows altered gustatory behavior and is defective in the neural pathway processing or discriminating gustatory information. Cloning of the *mvl* gene identified a protein highly similar to Nramp, which is expressed in neurons of the peripheral and central nervous systems and in macrophages (13). The yeast mutant *mif* is lethal and phenotypically expressed as a defect in import of certain proteins into the mitochondria (22). The *mif* mutant can be rescued by overexpression of two genes, *SMF1* and *SMF2*, which encode proteins related to mammalian Nramp.

These sequences were aligned by use of the PILEUP and MACAW programs (Fig. 1), and scores were calculated for pairwise comparisons (Table 1). Mammalian Nramp1 and -2 are 66% identical, animal sequences (Nramp1, Nramp2, and

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Abbreviation: TM, transmembrane.
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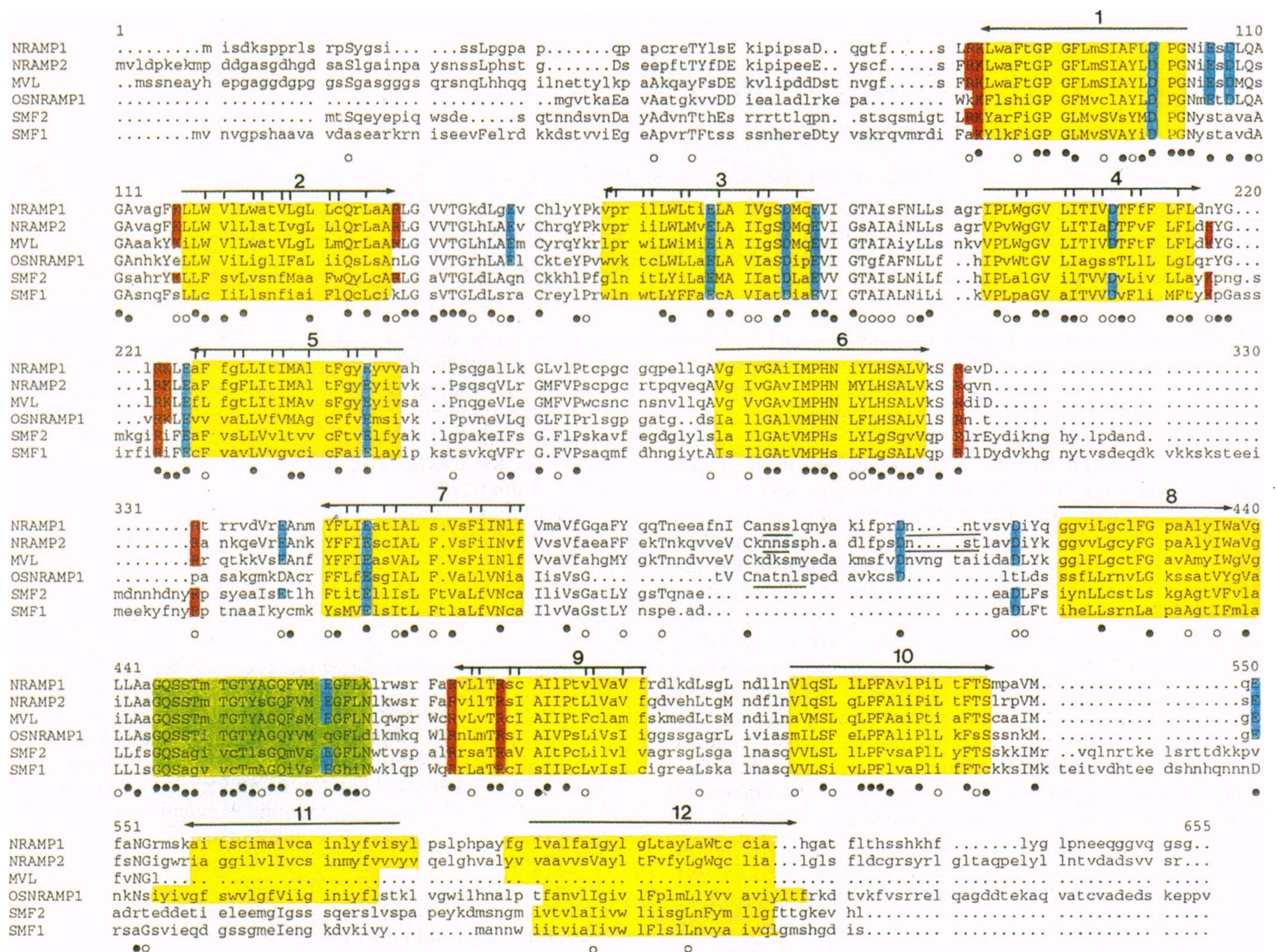

FIG. 1. Sequence alignment of Nramp-related polypeptides. The sequences of murine Nramp1 and Nramp2 (GenBank accession nos. L13732 and L33415), *Drosophila* Mvl (U23948), rice OsNramp1 (L41217), and yeast Smf1 and Smf2 (Swiss-Prot accession nos. P38925 and P38778) were aligned by the PILEUP program. Residues conserved in at least four of the six sequences are capitalized within the alignment and identical residues are further grouped according to the following code shown below the alignment: lower open circle (four of six), lower filled circles (four of four in animal and vegetal sequences), upper filled circles (six of six). Charges conserved in at least four of the six sequences are highlighted in red (+) and blue (−). The 12 putative TM regions are shown in yellow and highlighted by a line above the sequences which includes the orientation relative to the membrane (arrowhead, In). In some TM domains, residues located on a putative lipid-accessible face are identified by tick marks (only in animal and vegetal sequences for TM 7). Putative glycosylation sites are underlined and the putative transport-motif region is colored green.

Mvl) are at least 55% identical, and animal and plant (OsNramp1) sequences are 40% identical. The yeast Smf1 and Smf2 sequences share the same identity (52%) as animal sequences among themselves (Mvl vs. Nramp1 and -2). The global identity between Smf1/Smf2 and the other sequences is in the range of 25–30%. This high degree of conservation is remarkable for membrane proteins separated by such large evolutionary distances (up to 1 billion years), suggesting that together they may belong to a class of ancient membrane transporters. The highest degree of sequence conservation is within the hydrophobic core (in particular the amino and carboxyl boundaries) common to these proteins and forming the first 10 hydrophobic TM segments (Fig. 1, positions 81–529). Sequences in this core share 33–75% sequence identity, resulting in highly similar hydropathy profiles (Fig. 2). Outside the hydrophobic core, the similarity among the six proteins drops sharply. Amino termini are heterogeneous in length and very variable in sequence (Figs. 1 and 2), although a clustering of charged or polar residues is noted in this region for the six sequences. Likewise, the sequences of the carboxyl-

Table 1. Amino acid sequence relatedness between Nramp-related proteins

	Nramp1 (548 aa)	Nramp2 (568 aa)	Mvl (490 aa)	OsNramp1 (517 aa)	Smf2 (549 aa)	Smf1 (575 aa)
Nramp1	100	**66.4 (76.5)**	**54.9 (68.8)**	40.1 (**55.3**)	28.9 (42.1)	25.4 (46.3)
Nramp2		100	**57.4 (73.3)**	39.9 (**58.0**)	26.0 (44.3)	26.2 (46.2)
Mvl			100	36.2 (**54.5**)	29.0 (44.5)	29.0 (46.3)
OsNramp1				100	33.2 (46.6)	27.7 (47.2)
Smf2					100	**51.7 (65.2)**
Smf1						100

Percent identity and similarity (in parentheses) scores were calculated for each pairwise comparison based on the alignment of Fig. 1; scores above 50% are indicated in boldface.

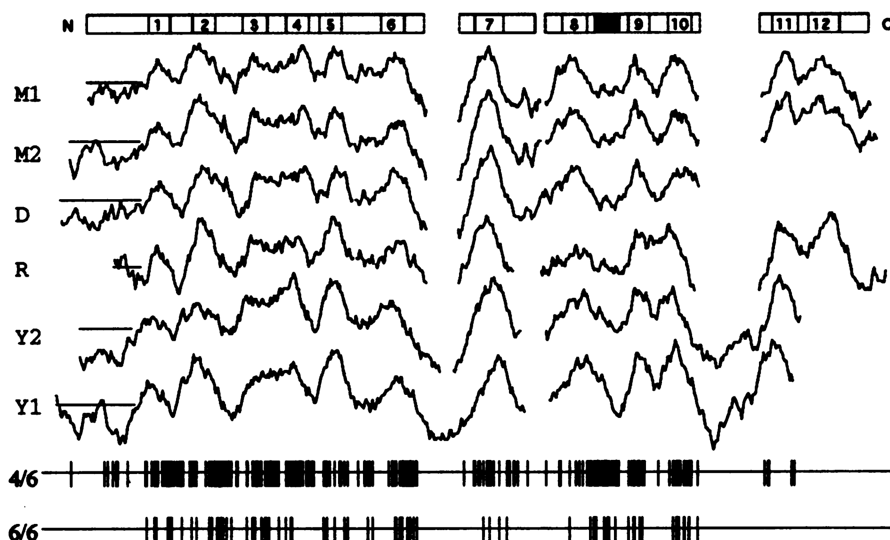

FIG. 2. Conservation of hydropathy profiles in Nramp-related sequences. (*Top*) Schematic representation of the Nramp protein identifying 12 predicted TM domains (numbered 1–12) and the consensus transport signature (filled box). (*Middle*) Alignment of hydropathy profiles (Kyte–Doolittle algorithm; window of 17). M1, mouse Nramp1; M2, mouse Nramp2; D, *Drosophila* Mvl; R, rice OsNramp1; Y2, yeast Smf2; Y1, yeast Smf1. (*Bottom*) Distribution of sequence identities (vertical bars) in either four of six or six of six sequences.

terminal regions are also not conserved; in particular Mvl is shorter, and Smf1 and Smf2 show quite distinct hydropathy profiles in this segment. These results suggest that the conserved hydrophobic core of Nramp-related sequences has been under strong evolutionary constraint to maintain a common structural or functional aspect of transport. This pattern of evolutionary conservation is reminiscent of that seen in ion transporters such as the amiloride-sensitive Na^+/H^+ exchanger family, where the human and worm proteins share 44% identity over a hydrophobic core including the TM 2–12 segment (23) and in ion channel families such as the voltage-gated K^+ channel (24).

Analysis of the Membrane-Associated Regions. The hydrophobic core of Nramp homologs was further analyzed for the presence, number, and structural characteristics of putative TM domains. TM segments were positioned by using hydropathy values, hydrophobic moment, minimal charge density, and the TMAP program (18). All methods independently offered very similar predictions, clearly identifying 10 TM segments within the common core and a maximum of 12 TM segments for the Nramp and OsNramp1 proteins (Figs. 1 and 2). TM domains 2, 5, 7, 9, 10, and 11 were the most hydrophobic and best delineated segments, while TM domains 3 and 4 were difficult to segregate within a broad hydrophobic region which also contained several charged/polar residues (Fig. 1). Predicted TM domains 1 and 6 were consistently associated with the lowest scores. The sequences were also analyzed by the MEMSAT program (19). Charge bias analysis is incorporated in this method, and predictions are made as to the overall optimal topology of the protein, including the direction of the TM helices through the membrane. This analysis predicted a consensus topology (Figs. 1 and 2) which placed the amino terminus intracytoplasmic, followed by 10–12 TM domains connected by hydrophilic segments, with the loop downstream from TM domain 10 positioned intracytoplasmic. MEMSAT analysis also delineated TM domain 4 but could not firmly position TM domain 3, which had the lowest score (data not shown). The inability to position TM domain 3 accurately by any method may be linked to the high charge density in this region of the six proteins. Finally, the carboxyl-terminal boundary of the hydrophobic core corresponds to the end of the polypeptide in Mvl, whereas Nramp1 and -2 and OsNramp1 contain two additional TM domains. In Smf1 and Smf2, this region displays a fairly broad hydrophobic peak capable of forming at least one and possibly two TM segments.

Analysis of sequence similarity within individual TM domains reveals a striking degree of conservation for TM domains 1, 4, 6, and 10, with 6 of 19 (TM 1), 8 of 20 (TM 6), and 6 of 19 (TM 10) residues invariant and 12 of 19 (TM 1), 15 of 20 (TM 4 and 6), and 12 of 19 (TM 10) residues identical in at least four of six sequences (Figs. 1 and 2). Interestingly, a $Gly^{169} \rightarrow$ Asp mutation in the highly conserved TM domain 4 causes a complete loss of function of Nramp1 (3, 8, 9). TM domains 8, 11, and 12 are the least conserved TM segments. Another remarkable feature of the hydrophobic core is the presence of several invariant charged residues within 6 of the 10 TM segments. TM domains 1 (Asp; six of six sequences), 3 (Glu and Asp; six of six sequences), 4 (Asp; five of six sequences), 5 (Glu; five of six sequences), and 7 (Glu; six of six sequences) contain negative charges whereas TM domain 9 contains an invariant positive charge (Arg; six of six sequences) (Fig. 1). The precise conservation of thermodynamically unfavored charged residues within TM segments strongly suggests that they play a major structural or functional role. In ionic channels and carriers, such conserved charged residues can mediate interactions with aqueous solvent or with substrate molecules, can monitor changes in channel environment (electrical potential), and can participate in gating/opening of a channel (24, 25). In other transporters (lactose permease), they may form ionic bridges important for interhelix packing (26). Helical wheel projections (Figs. 1 and 3) of the putative TM domains of the hydrophobic core reveal strong amphiphilicity for TM domains 3, 5, and 9 (and, to a lesser degree, 1 and 7), clearly segregating a polar face, enriched for charged/polar residues, and a nonpolar hydrophobic face. Examination of the periodicity in the sequence variability suggests that TM domains 1, 3, 4, 5, and 9 have an α-helical structure with a large variability moment. In the identified amphiphilic TM domains 3, 5, and 9, charged/polar residues cluster on the more conserved face of the helix, while the apolar "lipid-accessible" face of the helix is heavily substituted (Fig. 3). This striking feature suggests that the polar/charged faces of TM 3, 5, and 9 are key structural/functional components preserved during evolution.

Within the hydrophobic core, the sizes of certain intracytoplasmic and extracytoplasmic loops are found to be very conserved (TM 1–3 and TM 8–10 intervals) whereas others are not (TM 6–8 intervals) (Figs. 1 and 2). Sequence conservation

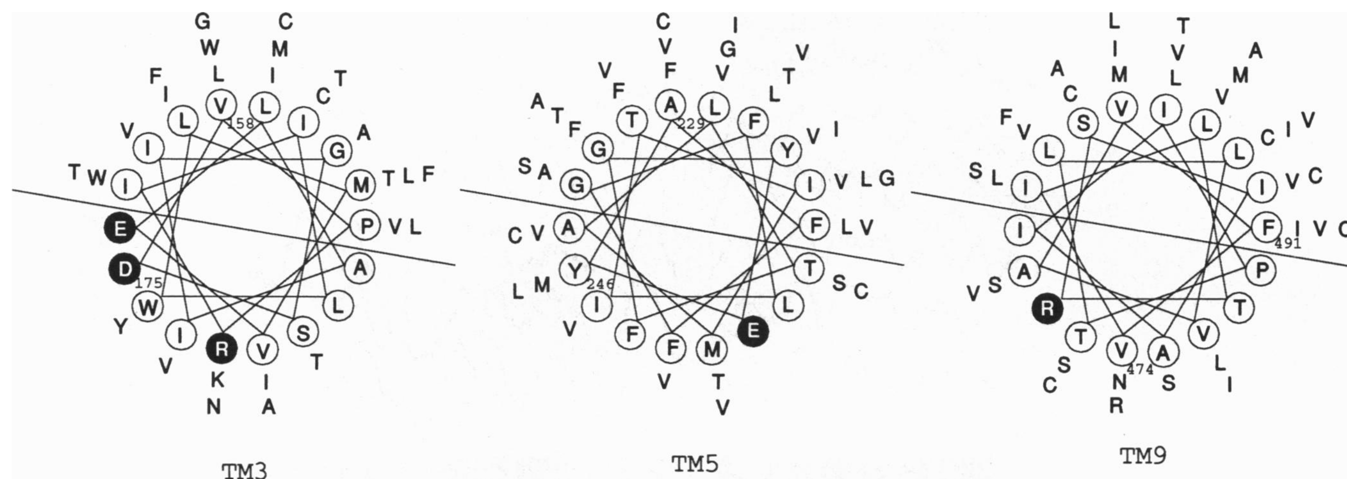

FIG. 3. Helical wheel projections of putative TM domains 3, 5, and 9 (18 residues). The sequence of Nramp2 was used as reference and is shown in circled residues, while substitutions in the five remaining sequences are indicated. Charged residues are identified by filled circles. In all three projections, the face below the horizontal line is both more polar/charged and more conserved and may contact other helices. The nonpolar and more variable face is compatible with exposure to the lipid bilayer (21).

within the loops is also restricted to particular areas—e.g., the short TM 1–2, 2–3, 3–4, and 8–9 intervals. Conversely, TM domains 6, 7, 9 and 10, while displaying a high degree of sequence conservation, are flanked by highly variable segments. This pattern of conservation is once again reminiscent of that seen in families of ion channels, where conservation of sequences outside the TM domains seems to underlie a common architecture of the inside or outside vestibules of the channels, while other highly variable loops may reflect differences in either regulation or specificity of substrates and/or sensitivity to isoform-specific inhibitors (27). Another noticeable feature of intervening loops in the core is a remarkable excess of positive charges on the intracytoplasmic side (Fig. 1). Calculation of net charges for the loops defined by TM 2–3, 4–5, 6–7, and 8–9 intervals gave positive scores of +9 (Nramp2), +8 (Mvl), +7 (Nramp1, OsNramp1), +6 (Smf1), and +1 (Smf2). Such clustering of positive charges has been proposed to play a role either in membrane targeting or in TM domain orientation/topology (for review, see ref. 28) or to produce surface charge effects possibly aimed at increasing substrate concentration at the site of transport (29). Within the 10 TM domains of the hydrophobic core, a net charge imbalance of −4 is noted in all sequences except for OsNramp1, with -2, and remarkably, two-thirds of these charges are absolutely conserved in the six sequences. Such an excess of negative charges has been noticed in several transporters, including the Cl^-/HCO_3^- anion exchanger (30). Finally, we noted an excess of negative charges of −7 (Mvl), −5 (Nramp2, OsNramp1), −3 (Nramp1), −2 (Smf1), and −1 (Smf2) in the predicted extracytoplasmic loops. Therefore, the predicted consensus topology of the hydrophobic core results in an asymmetric distribution of charges with a strongly positive interior, a negative transmembrane region, and a negative extracytoplasmic region.

Additional Conserved and Nonconserved Sequence Motifs. A sequence motif known as the "binding-protein-dependent transport system inner membrane component signature" [(E, Q)(S, T, A)$_2$X$_3$GX$_6$(L, I, V, M, Y, F, A)X$_4$(F, I, L, V)(P, Q); ref. 3] was found within the highly conserved TM 8–9 intracytoplasmic loop (Fig. 1). This motif was originally identified within cytoplasmic domains of membrane components of bacterial traffic ATPases (6), where it was proposed to mediate coupling of the peripheral ATP-binding units to the membrane components of these transporters (31). This signature has since then been detected in at least 51 prokaryotic and eukaryotic membrane proteins (11). Although the precise role of this sequence motif remains unclear, mutations near or at this site abrogate function (32). Intriguingly, this segment (positions 442–463, Fig. 1) presents an independent similarity (including an invariant Gly-Gln at positions 456 and 457) to a sequence motif identified in the K^+ channel superfamily as a key structural determinant of the ion permeation pathway and shared with other ion channels and transporters (25). The presence of a glycosylated loop in the TM 7–8 interval has been preserved in all but the Smf1 and Smf2 sequences, pointing to an important role of glycosylation of this loop in proper targeting or processing of the polypeptides. Finally, putative phosphorylation sites (3) and Src homology 3 (SH3) binding domain previously detected in Nramp1 (33) are not conserved. This suggests either a lack of functional importance or differences in regulation of these proteins.

Structural and Functional Implications. We propose that the identified Nramp homologs form a family of structurally and probably functionally related membrane proteins. The structural basis of this family is the presence of a common conserved hydrophobic core (33–75% identity) encoding 10 highly conserved TM segments, with additional similarity clustered in certain intracytoplasmic and extracytoplasmic loops. Such a striking degree of evolutionary conservation within the core suggests that it underlies a key function common to these proteins. A more detailed analysis of the 10 TM segments reveals unique features reminiscent of ion transporters and channels. Indeed, most TM segments when projected in a α-helical configuration segregate a conserved and a substituted face. For three amphipathic predicted helices, the conserved face contains invariant polar/charged residues, whereas the more substituted one is very hydrophobic. These structural predictions are compatible with an α-helical-bundle structure for the hydrophobic core (20). The lipid-accessible face of this bundle would be formed by the less conserved side of helices (on the exterior), while the more conserved face, containing invariant hydrophobic and polar/charged residues, would form the interior. This would enable either interhelix interactions or accessibility to aqueous solvent and possibly interaction with a hydrophilic or charged substrate inside the core (30), as observed on the hydrophobic core of Cl^- and K^+ channel families (24, 34). The less conserved hydrophobic exterior could reflect adaptation to different lipid environments or less stringent structural requirements to maintain lipid solubility and membrane insertion. The lack of sequence conservation outside this core may

reflect differences in either regulation of the same transport event or differences in substrate specificity.

A review of the genetic and biochemical characterization of Nramp-related proteins may be appropriate in view of a proposed common mechanistic aspect of ion transport. *Nramp1* is expressed exclusively in professional phagocytes and may be involved in modifying the intracytoplasmic milieu of these cells to suppress replication of unrelated intracellular parasites. Since transit through the phagosome is shared by these parasites, Nramp1 may be expressed in the membrane of these vesicles to modify the intraphagosomal environment and thus inhibit microbial replication. The $Gly^{169} \rightarrow$ Asp replacement within the highly conserved TM domain 4 abrogates Nramp1 function and results in uncontrolled intracellular replication. The role of *Nramp2* (11, 12) remains obscure, as it is ubiquitously expressed and no mutants are available to assess the consequence of loss of function. Although the function and substrate of OsNramp1 remain unknown, it is expressed in the root system and belongs to a highly conserved family of plant genes (36). Taste discrimination in flies involves two types of neuronal signals: an afferent one from gustatory receptors, which provides sensory information to the brain, and an efferent one, which involves integration and processing of the gustatory information and triggers appropriate behavioral modification. The malvolio mutation affects the latter pathway. The *mvl* gene is expressed in mature neurons of the central and peripheral nervous systems, and it is easy to conceive how alterations in an ion channel which may be important for signal processing by neurons may account for the *mvl* defect (13). Finally, although the biochemical basis of Smf function is unknown, combined but not single mutations at *SMF1* or *-2* cause reduced protein import into mitochondria and severe growth retardation. The insertion of an epitope tag within predicted intracytoplasmic loops of the hydrophobic core (TM 6–7; TM 8–9) abrogates the capacity of *SMF1* to suppress *mif*, pointing at an important role of these loops in Smf protein function. Interestingly, *SMF1* and *SMF2* can complement a temperature-sensitive allele, but not a null allele, of *mif*, suggesting an indirect compensatory mechanism caused by *SMF1* or *SMF2* overexpression. Since an intact membrane potential is required for efficient protein import into mitochondrial matrix (35), the possibility exists that Smf1 and -2 may modulate this process. Although a discussion of such proposed functions is inherently speculative in the absence of detailed biochemical studies, the identification of a common phylogeny for these genes provides a rational basis to assay their function in complementation studies in their respective systems.

Note. While this paper was under review, three additional Nramp-related sequences from *Caenorhabditis elegans* (2) and *Mycobacterium leprae* (GenBank accession nos. U23525 and U15184, respectively) were identified. The two *C. elegans* sequences show 74.6% identity to each other, and are 56–61% identical to Mvl, 53–58% identical to mammalian Nramp1 and -2, and 39–43% identical to OsNramp1. The *M. leprae* hypothetical polypeptide sequence displays 31% (yeast Smf1) to 37% (Nramp1) sequence identity. The major structural features of Nramp-related sequences identified in this paper are also precisely conserved in the *C. elegans* and *M. leprae* sequences (hydrophobic core, TM domains, and transport motif).

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