Brief Definitive Report

NH_2-Terminal Sequence of Macrophage-expressed Natural Resistance-associated Macrophage Protein (*Nramp*) Encodes a Proline/Serine-rich Putative *Src* Homology 3-binding Domain

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Summary

The *Lsh/Ity/Bcg* locus on mouse chromosome 1 regulates macrophage (mϕ) priming/activation for antimicrobial activity against intracellular pathogens. A candidate *Bcg* gene, designated natural resistance-associated mϕ protein (*Nramp*), recently isolated from a pre-B cell cDNA library encodes a polytopic integral membrane protein with structural features common to prokaryotic and eukaryotic transporters. In the present study, an activated mϕ cDNA library yielded new *Nramp* clones that differ in the 5′ region from the published pre-B cell–derived clone sequence, resulting in addition of 64 amino acids at the NH_2 terminus of the predicted protein. This new domain is rich in proline, serine, and basic amino acids, and includes three protein kinase C phosphorylation sites and a putative Src homology 3 binding domain. RNAs containing this domain are the only form found in the mϕ. Hence, the protein encoded by this RNA is the candidate molecule mediating natural resistance to intra-mϕ pathogens.

The murine macrophage (mϕ) resistance gene *Lsh/Ity/Bcg* regulates the priming/activation of mϕ for antimicrobial activity against *Leishmania donovani*, *Salmonella typhimurium*, and *Mycobacterium spp.* (1–5). Recently Vidal et al. (6) isolated a candidate gene, designated *Nramp* (natural resistance-associated mϕ protein), that encodes a polytopic integral membrane protein that has structural features common to prokaryotic and eukaryotic transporters. The presence of a small consensus motif showing sequence identity with nitrate transporters led these workers to hypothesize that *Nramp* might function as a nitrate concentrator in the phagolysosome membrane of the infected mϕ, the acid environment of the phagolysosome mediating conversion via nitrite to toxic nitric oxide (NO). However, this hypothesis fails to take account of many studies demonstrating that *Lsh/Ity/Bcg* regulates mϕ activation (7–10) leading to the TNF-α–dependent production of antimicrobial NO (11). The gene also has many pleiotropic effects including: (*a*) downregulation of 5′nucleotidase (9, 10); (*b*) upregulation of MHC class II, TNF-α production, IL-1β expression, AcM.1 antigen expression, oxidative burst, and tumouricidal activity (7–10); (*c*) rapid (within 30 min) upregulation of the early gene KC, a neutrophil-specific chemoattractant of the IL-8–related C-X-C family of small peptide cytokines (12); and (*d*) integrin-mediated upregulation of TNF-α production by plating mϕ onto the extracellular matrix proteins fibrinogen and fibronectin (13). These observations suggest that *Lsh/Ity/Bcg* must encode a molecule that plays a role early in the mϕ activation pathway leading to antimicrobial activity. A study of mϕ-expressed *Nramp* was therefore undertaken to determine how gene structure might relate to these functional observations and whether *Nramp* is a valid *Lsh/Ity/Bcg* gene candidate.

Materials and Methods

Sequence Analysis of Nramp Clones from mϕ cDNA Library. Mϕ*Nramp* clones were isolated from an activated (4 h stimulation; 25 U/ml interferon-γ, 10 ng/ml *S. typhimurium* LPS) mouse (B10.L-*Lsh*^{r}) mϕ cDNA library prepared in λ UniZap (Stratagene, La Jolla, CA). Clones were isolated by filter plaque hybridization using a probe generated by reverse transcriptase–PCR corresponding to nucleotides 1410–1812 bp of the published (6) sequence. After plaque purification, 35 clones from 10^6 recombinants were analyzed by PCR using sense and antisense *Nramp* primers in combination with T3 and T7 vector arm primers. This allowed the mapping of clones with respect to the published sequence. 20 out of 30 were 1.0–1.5 kb and were not analyzed further. The remainder were 2.1–2.3 kb and potentially full-length *Nramp* clones. These were restriction mapped and four were selected for sequencing (Sequenase II) including the longest clone λ8.1.

Genomic Sequencing. From the mϕ sequence, PCR primers (CCT GGT GAC CAC ACA CAG and CAC CTT GGG GTA GAG ATG) were generated to amplify a 2-kb region from both yeast artificial chromosome (YAC clone C9C28; Princeton library, Howard Hughes Medical Institute, Princeton University, Princeton, NJ) and mouse genomic DNAs. The products were cloned in the

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pCR vector (Invitrogen, San Diego, CA) and sequenced (Sequenase II) from double stranded plasmid DNA from at least two clones of each using primers complementary to the cDNA sequence. Splice junctions were identified by comparison of the genomic and cDNA sequences. Genomic sequence was also obtained across exon 2 of the human NRAMP gene using an 18-kb EMBL3 clone isolated from subcloned human YAC clone AM11/D3/14 (Imperial Cancer Research Fund library, London, UK). This YAC was shown by fluorescence in situ hybridization (FISH) to hybridize to the region of human 2q35 homologous with murine chromosome 1.

Northern Blot and Primer Extension Analysis of Nramp Expression. Cytoplasmic total RNA isolated in the presence of vanadyl ribonucleoside complexes was used for denaturing gel electrophoresis with glyoxal and Northern blotting, or directly for reverse transcriptase reactions for primer extension analysis. Hybridizations were performed using probes derived from a PCR product (primers CCT GGT GAC CAC ACA CAG and TGC AAG CAG ATC GGG TCA) covering the genomic sequence (base pairs 1–1482) 5′ of exon 3 (see Results and Discussion). Restriction digestion with BamHI generated two probes covering a λ8.1-specific (= base pairs 1–587 of the genomic sequence; see Fig. 2 *b*) region or the putative 5′ untranslated (UTR) sequence (base pairs 588–1482 of the genomic sequence; see Fig. 2 *b*) of the published (6) cDNA. Southern blotting (not shown) confirmed that both probes hybridized to EcoRI fragments of 3,500 and 500 bp in mouse genomic DNA from both C57BL/10ScSn (*Lsh*^{s}) and B10.L-*Lsh*^{r} mice. For primer extensions oligonucleotides designed to be complementary to the λ8.1-like RNAs (TCT GCG CTG GGA ATG GGG; base pairs 538–521 of the genomic sequence) or complementary to the putative 5′ UTR of the published sequence (TGC AAG CAG ATC GGG TCA; base pairs 1,482–1,465 of the genomic sequence), were labeled with polynucleotide kinase. Extension reactions were performed with 25 U AMV reverse transcriptase at 42°C, terminated by addition of gel loading buffer, and sized against a sequencing ladder after denaturing polyacrylamide gel electrophoresis.

Computer-assisted Analysis. Hydropathy profiles of the predicted NH_2-terminal amino acid sequence of mϕ-expressed *Nramp* were obtained by computer-assisted analysis using the algorithm and hydropathy values of Kyte and Doolittle (14). Amino acid sequence comparisons (15) were made using the FASTA programme on-line to the Clinical Research Centre Resource Centre (Harrow, UK).

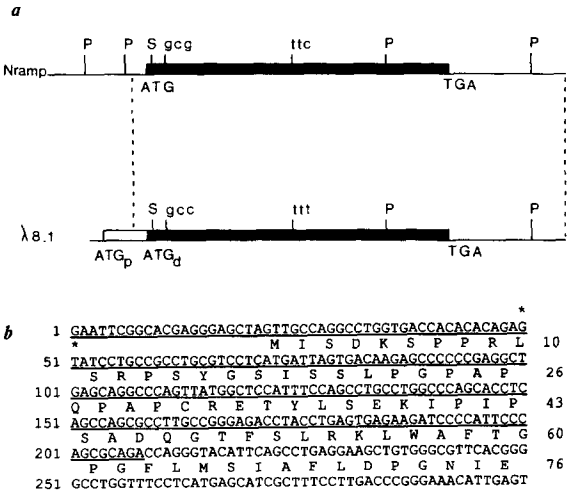


Figure 1. Alignment of pre-B cell (6) and mϕ-expressed (λ8.1) *Nramp*. (*a*) Map showing regions of sequence identity (*broken lines*) between the published clone and mϕ clone λ8.1, including the ATG (*d*) codon and the major ORF (*solid bars*) of published *Nramp*. Positions of SmaI (*S*) and PvuII (*P*) cleavage sites demonstrate 5′ divergence between clones. Novel 5′ sequence in λ8.1 contained a proximal ATG (*p*) codon and an extended ORF (*open bar*) encoding 64 NH_2-terminal residues not present in published *Nramp*. (*b*) 5′ sequence of mϕ λ8.1 (EMBL Accession Number X75355) encoding the 64 NH_2-terminal residues 5′ of the distal initiation codon (Met at position 65) of Vidal et al. (6). The remaining sequence (not shown) was identical to the published clone except for two silent mutations. (*asterisk*) Sites of transcriptional initiation determined by primer extension analysis (see Fig. 3).

Results and Discussion

Sequence Analysis of mϕ-derived Nramp cDNA Clones. Screening an activated mϕ library yielded 15 full-length *Nramp* cDNAs, 14 of which differed from published (6) *Nramp* in the 5′ terminal sequence. The longest mϕ-derived cDNA (Fig. 1; λ8.1) was 186 bp shorter than *Nramp*. It contained the full-length coding region for the previously predicted protein and was identical over the coding region of the published (6) sequence except for two silent mutations (359 bp, C; 965 bp, T). It also exhibited 100% identity with no in-frame stop codons for the region (base pairs 209–263) of putative UTR sequence immediately 5′ of the published initiation codon. However, nucleotides 1–208 of λ8.1 shared no identity with published *Nramp*. A more proximal ATG codon was identified at 72 bp in λ8.1, preceded by an in frame stop codon at 36 bp. This proximal translational initiation codon is followed by an open reading frame (ORF) of 192 bp (64 amino acids) that leads into the ORF previously reported. Previous studies show that proximal initiation codons are used in >90% of genes analyzed (16). Nor was there any evidence that the distal initiation codon would be favored, since both distal and proximal initiation codons and flanking sequences are identical (TCCTCATGA) and display only two identities with the optimal (17) ($CC^A/_GCCATGG$) consensus. Hence, there is no a priori reason why the proximal initiation codon would not be used. The additional 64–amino acid sequence is identical in resistant and susceptible mice (data not shown), is rich in Ser 10/64, Pro 10/64, basic 7/64 residues, and contains three consensus protein kinase C phosphorylation sites (S/T-X-R/K) on Ser 3, 37, and 52. Database searches also revealed a B-2 Alu-like repetitive element (not shown) within the 3′ UTR, which produces complex signals when the full-length λ8.1 clones is hybridized to mouse genomic DNA.

Genomic Sequence for the 5′ Region of Nramp. To determine whether mechanisms exist that could generate two RNAs and hence two types of *Nramp* clones, a region of genomic DNA spanning the divergence was characterized corresponding to nucleotides 31–456 of λ8.1 (Fig. 2). This region is encoded by four exons interspersed by three introns of 395, 900, and 241 bp, with all splice donor and acceptor sites conforming to the GT and AG boundaries. The first 47 amino acids of the 64 amino acid NH_2-terminal domain of λ8.1 are encoded by two proximal exons unique to this clone. The remaining 17 amino acids are encoded by exon 3, with exons 3 and 4 common to both λ8.1 and the pub-

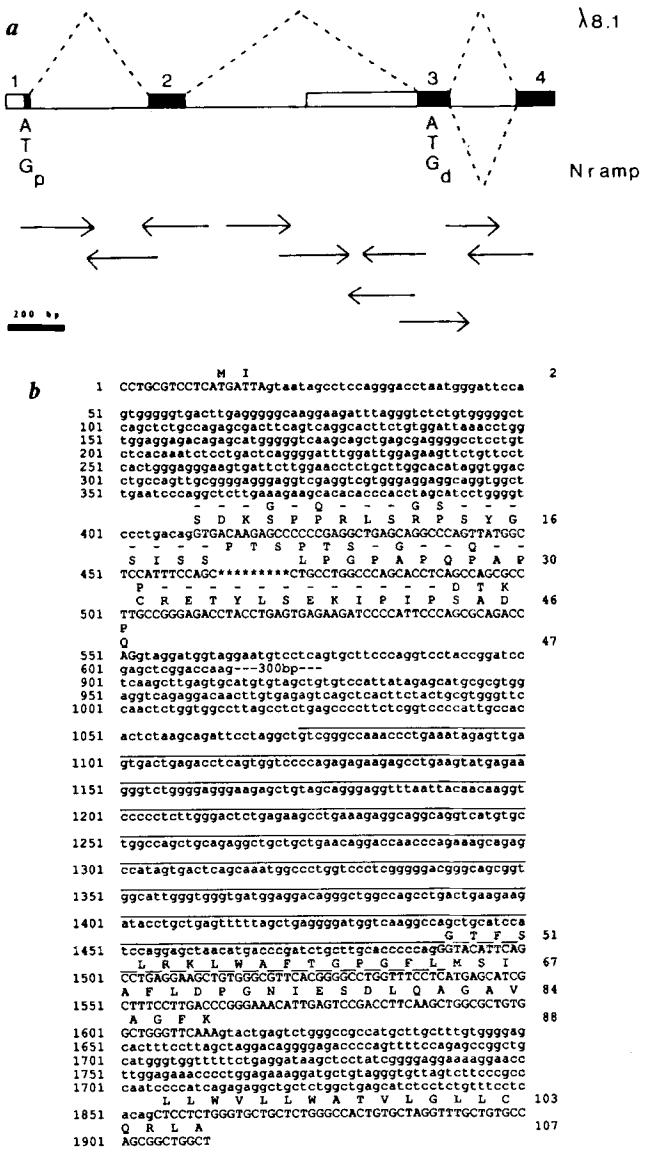


Figure 2. Map and genomic sequence for the 5′ region of *Nramp*. (*a*) Map demonstrating that the additional sequence of mϕ *Nramp* is encoded by two unique exons (1 and 2; *solid bars*) contiguous in the cDNA sequence (Fig. 1) with exons (3 and 4; *solid bars*) common to both λ8.1 and published *Nramp*. Contiguous with and 5′ of exon 3 is the putative 5′ UTR (*open bar*) found earlier (6). Predicted splicing patterns (*dotted lines*) are indicated above (λ8.1) and below (published pre-B cell clone) the map. Arrows indicate primers for sequencing gel reads. (*b*) Genomic sequence corresponding to nucleotides 31-456 of λ8.1 spanning the point of divergence with the published (6) sequence. Exonic nucleotide sequence is in capitals, with the predicted amino acid sequence above in single letter format. Intron sequence is in small letters. The region of 5′ UTR from the published clone, contiguous with exon 3, is overlined. The codon (ATG = Met) where this terminates indicates the initiation codon of published *Nramp*. The predicted amino acid sequence for human NRAMP exon 2 is shown above the murine sequence. (−) amino acid identity. (*asterisk*) Gap introduced for alignment of human and murine sequences.

lished (6) *Nramp* cDNA. The 5′ UTR sequence from the published clone was found in the 900-bp intron contiguous with and including part of exon 3, indicating that a single gene encodes both forms. Exon 3 is particularly unusual in that it encodes protein sequence in λ8.1, whereas for published *Nramp* it contains both coding and noncoding sequence. Although a complex mechanism involving alternative splicing with an internal splice acceptor site and dual promoter control could be formulated to account for both forms, it seems more likely that the published (6) cDNA clone contains a fragment of the 900-bp intron at its proximal end. This is consistent with the observation that a number of the mϕ-derived *Nramp* clones isolated here were found to contain sequence that exhibited identity with the first *Nramp* intron identified in genomic DNA (not shown). The human genomic sequence corresponding to base pairs 408–455 of the murine genomic sequence (Fig. 2) shows 77% identity with mouse at the nucleotide level (not shown) and 68% (82% with conserved substitutions) for the predicted amino acid sequence (Fig. 2). The predicted amino acid sequence encodes a similar proline/serine-rich domain containing an additional 3 amino acid insert at position 21 of the murine sequence.

Only One Form of Nramp Is Expressed in mϕ. To confirm that RNA encoding the longer polypeptide is the form expressed in mϕ, a number of experimental approaches were adopted (Fig. 3). Using mϕ RNA as template, primer extension with an oligonucleotide unique to the 5′ region of λ8.1 yielded products in both susceptible and resistant mice mapping to nucleotides 50/51 of the cDNA sequence (Fig. 1 *b*). In contrast, no products were generated using an oligonucleotide within the putative 5′ UTR of published (6) *Nramp*. These experiments confirmed that RNA transcripts bearing the putative 5′ untranslated region of the published cDNA are not present in resting (not shown) or activated mϕ, whereas transcripts corresponding to the λ8.1 sequence were identified with transcriptional initiation sites mapping 21 and 22 bp (doublet) 5′ of the proximal ATG codon. A probe covering the 5′ region unique to λ8.1 also hybridized well to Northern and slot blots of macrophage RNA from susceptible and resistant mice, whereas a probe covering the putative 5′ UTR of the published clone showed no hybridization. Hence, the only form of RNA transcript present in mϕ is that which conforms to the λ8.1 predicted polypeptide sequence. It is therefore this polypeptide that is the candidate natural resistance protein.

Predicted Structure and Sequence Identities Across the NH_2-terminus of mϕ-expressed Nramp. To determine how the additional domains of mϕ-expressed *Nramp* influence the structure of the molecule, hydropathy (14) plots, and amino acid database searches were undertaken over the 64-amino acid domain. The former (data not shown) demonstrated that the new sequence is hydrophilic, and forms an extension to the NH_2-terminal cytoplasmic domain. The amino acid database search (15) over this proline, serine and basic rich 64-amino acid domain identified three PKC phosphorylation sites (in addition to two identified in published *Nramp*), and a number of matches with unrelated proteins (Fig. 4). The most in-

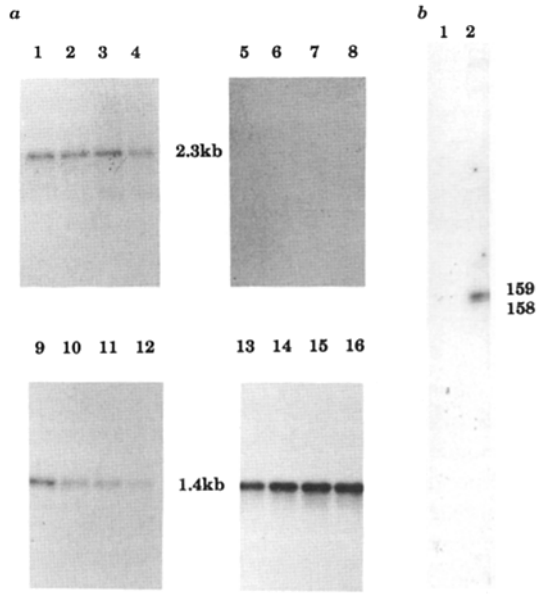
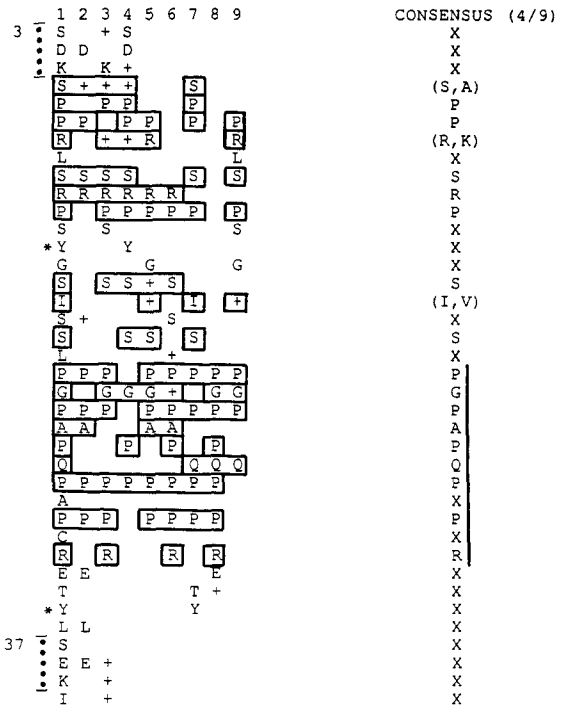


Figure 3. Northern blot (*a*) and primer extension (*b*) analysis demonstrating that *Nramp* transcripts encoding the additional 64 amino acids are the only form of *Nramp* expressed in mϕ. (*a*) Northern blots from resting mϕ (lanes *1*, *5*, *9*, and *13*), or mϕ activated with interferon-γ (lanes *2*, *6*, *10*, and *14*), LPS (lanes *3*, *7*, *11*, and *15*), or interferon-γ plus LPS (lanes *4*, *8*, *12*, and *16*) were hybridized with probes specific for unique λ8.1 5′ sequence (lanes *1–4*), or for the more distal putative 5′UTR of the published (6) sequence (lanes *5–8*). Lanes *9–16* show the same blots reprobed with GAPDH. Twice as much RNA was loaded on blots hybridized with the distal probe. Results are shown for RNA extracted from bone marrow-derived mϕ from C57BL/10ScSn mice. Slot blot analysis (not shown) confirmed that the λ8.1-specific probe hybridized to RNA from both susceptible and resistant macrophages. (*b*) Primer extension products obtained with 10 μg of total RNA from B10.L-*Lsh*^{r} mϕ using oligonucleotides specific to the putative 5′ untranslated region of the published (6) sequence (lane *1*), or to 5′ sequence unique to λ8.1 (lane *2*). The numbers of nucleotides from the 5′ end of the primer are shown. Control reactions with tRNA gave no products with either primer (not shown). Similar results were obtained using RNA from C57BL/10ScSn (*Lsh*^{s}) mϕ as template.



1. NRAMP CLONE 8.1 DERIVED SEQUENCE
2. NIF-SPECIFIC REGULATORY PROTEIN 12/35 IDENTITIES (34%)
3. GRANULINS PRECURSOR (AGROGRANIN) 13/38 IDENTITIES (34%)
4. FAK PROTEIN TYROSINE KINASE 13/26 IDENTITIES (50%)
5. TEGUMENT PROTEIN UL49 12/23 IDENTITIES (52%)
6. BETA-1-ADRENERGIC RECEPTOR 12/21 IDENTITIES (57%)
7. EXTENSIN (PRO RICH GLYCOPROTEIN) 14/30 IDENTITIES (46%)
8. EBNA-1 9/13 IDENTITIES (69%)
9. DYNAMIN (SHIBIRE PROTEIN) 11/20 IDENTITIES (55%)

Figure 4. Results of amino acid database searches with the 64 amino acid sequence unique to macrophage-expressed *Nramp* identifying a number of sequence matches particularly with the proline rich sequence; + represents a conserved amino acid. Multiple sequence alignments allowed for the generation of a consensus motif over this region: PGPAPQPXPXR (*solid vertical bar*). The nine best matches were aligned with each other and residues boxed where four or more exhibited identities. Also shown are the two PKC sites (*hatched vertical bars*) on S3 and S37 which flank the region exhibiting sequence identity. Tyrosine residues (*asterisk*) occur on either side of the consensus motif.

triguing matches were with: (*a*) *Drosophila* dynamin (18) (55% identity over 20 residues), related to the synaptic phosphoprotein dephosphin in rat brain (19); (*b*) the proline-rich third cytoplasmic domain of the adenylate cyclase stimulatory and G protein coupled β1-adrenergic receptor (20) (57% over 21 amino acids); and (*c*) focal adhesion kinase (21) (50% over 26 amino acids) modulated by integrin-dependent phosphorylation (22). The region of identity with dynamin has been implicated (23) in binding anionic phospholipids, microtubules and Src homology 3 (SH3) domains. SH3 domains (24, 25), identified as related sequences in tyrosine kinases (TK), are modular and found in proteins such as non-receptor TKs, phospholipase C-γ and structural proteins of the cytoskeleton. Whilst the function of SH3 domains (24) is not as well characterized as the SH2 counterpart, it is believed they mediate specific protein–protein interactions obligatory for signal transduction (25). Members of the Src family of membrane-associated TKs, including Hck and Fgr which have SH3 domains, are also found in mϕ (26). Both exhibit differential kinetics in response to activation signals and could be implicated in *Nramp*-mediated signal transduction. Hck, in particular, is involved in signal transduction for TNF-α production in murine mϕ (27), which is crucial for NO production and antimicrobial activity in *Lsh*-resistant mϕ (11). Early gene KC expression in *Lsh*-resistant mϕ also involves a Ca^{2+}-dependent, NO-mediated, cyclic GMP-dependent kinase pathway (12). Phosphorylation of the *Nramp* SH3-binding domain on tyrosines might itself regulate transport of important substrates (e.g., L-arginine) required initially for generation of NO mediating signal transduction (12) and subsequently for antimicrobial activity (11). Bogle et al. (28) have shown that L-arginine transport by mϕ is stimulated upon activation with interferon-γ and LPS, providing a mechanism for sustained substrate supply during the generation of NO. Control of transport function through interaction of TKs with the putative SH3 binding domain of *Nramp* may

provide a link between its structural identity with other transporters (6) and the decade of functional analysis demonstrating a role for the *Lsh/Ity/Bcg* gene in mϕ activation (7–13). The pleiotropic effects of *Nramp* would thus be due to its role in providing the substrate essential for generation of NO involved in signal transduction and as the final effector for antimicrobial activity. Further analysis of mϕ-expressed *Nramp* provides an exciting basis to future research aimed at understanding mϕ activation at the molecular level.

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