***Nramp1* locus encodes a 65 kDa interferon- γ -inducible protein in murine macrophages**

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The murine *Nramp1* (natural-resistance-associated macrophage protein) locus, formerly known as *Ity/Lsh/Bcg*, was isolated previously on the basis of chromosomal location, and as conferring natural resistance to infection against intracellular macrophage pathogens. The gene encodes a transporter molecule of unknown function. We have prepared polyclonal antisera against the C-terminal 35 amino acids of murine Nramp1. This serum is reactive towards a 65 kDa protein, expressed in murine macrophage cells from resistant or susceptible mice stimulated with interferon- γ and lipopolysaccharide, but not in non-macrophage cells. Evidence indicates that Nramp1 is localized in a subcellular membrane rather than at the cell surface. This evidence includes:

the identification of conserved endocytic targeting motifs following inspection of human and murine Nramp sequences; the enrichment of Nramp1, following magnetic selection of phagolysosomal vesicles from activated macrophages that were allowed to phagocytose magnetic, IgG-coated beads; confocal microscopy. These studies place Nramp1 on a membrane in close proximity to obligate intracellular pathogens. A link between Nramp1 and divalent-cation transport is suggested by sequence similarity with yeast SMF1. Evidence showing modulation of Nramp1 protein levels by iron chelation provides a direct link with Nramp1 function and divalent-cation metabolism.

INTRODUCTION

A single locus in mouse called *Lsh/Ity/Bcg*, mapping to the proximal region of chromosome 1, determines the innate immune response towards a number of obligate intracellular macrophage pathogens, including *Leishmania donovani*, *Salmonella typhimurium*, *Mycobacterium lepraemurium*, *Mycobacterium bovis* and *Mycobacterium intracellulare* [1–3]. *Lsh/Ity/Bcg* is expressed from one of two alleles, termed resistant or susceptible, and which influence the proliferation of parasites in the reticulo-endothelial organs during early infection. A positional cloning approach leading to gene isolation [4] and subsequent full-length sequence identification [5a] was adopted to define the molecular basis for the two alleles and the nature of the encoded polypeptide. A gene termed *Nramp1* (natural resistance-associated macrophage protein), mapping proximal to *Il8r* and *Vil* [6] genes, was proposed as a candidate on the basis of appropriate expression characteristics, suitable map location, non-conservative polymorphism involving a Gly^{resistant} to Asp^{susceptible} transition and sequence identity with a transporter of nitrate from *Aspergillus nidulans* and with reactive nitrogen oxides being intimately linked with the mechanism responsible for pathogen killing [5a]. The *Nramp1*-encoded polypeptide (Nramp1) has a strikingly hydrophobic character with as many as 10 and possibly 12 membrane-spanning helical domains typical of many transporter molecules from both prokaryotes and eukaryotes. The region of the second or fourth membrane-spanning helix is well conserved and is the location of the susceptibility-associated mutation. In addition, the protein displays a well-conserved sequence motif resembling a consensus transport signature found in several prokaryotic and eukaryotic membrane-transport proteins and linking transport with energy utilization. *Nramp1* also displays several conserved

sites between mouse and human sequences for phosphorylation by protein kinase C, a glycosylated loop domain and an N-terminal domain rich in proline and hydroxylated (serine and threonine) amino acids. The latter has been proposed to encode a possible SH3-binding domain, although this region, together with the C-terminal, display poor conservation of sequence between human and mouse. Direct proof that the *Nramp1* locus encodes the susceptibility gene has been provided by several lines of evidence, including haplotype mapping [7], gene targeting [8], transgenic mouse technology [9] and biallelic cDNA transfection [10]. The latter technique is possible as a result of a large number of so-called pleiotropic effects associated between either resistant or susceptible allele-expressing cells that are thought to be manifestations of *Nramp1* gene function in cell signalling pathways.

The Nramp1 polypeptide defines a new family of membrane-associated proteins that display a high sequence conservation from yeast to human and with many species expressing at least two discrete gene copies [11]. Aside from the original hypothesis that Nramp1 displays a transport activity for reactive nitrogen intermediates into the phagolysosome [5], other clues as to the transport substrate have come from studies on the structurally similar genes to Nramp1 found in yeast, SMF1 and SMF2 [12]. SMF1 and 2 were isolated by using a complementation assay, with the isolated Nramp1 structural homologues being able to suppress a temperature-sensitive mutation in the processing enhancement peptidase (PeP) associated with protein translocation into the mitochondria. More recent studies [13] have questioned these original claims on the function of the yeast SMF genes. In one study, a novel mutant, unable to grow in the presence of 12.5 mM EGTA, was produced by replica plating. This novel phenotype is caused by a single amino acid change

Abbreviations used: IFN- γ , interferon- γ ; MBP, maltose-binding protein; LPS, lipopolysaccharide; NF-IL-6, nuclear factor interleukin-6.

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G149R in the essential yeast gene *CDCl*, with suppression being provided by overexpression of a high-copy *SMF1* gene. *SMF1* displays 55% sequence similarity with *Nramp1* [14] and its deletion in yeast renders cells sensitive to low manganese concentrations, inferring that *SMF1* encodes a high-affinity manganese transporter. Antibodies raised to the product of the *SMF1* locus demonstrated its presence in the yeast outer membrane. It has been proposed that elevated intracellular concentrations of manganese, brought about by overexpression of the *SMF1* gene or maintenance of cells in elevated manganese, enable the temperature-sensitive mutation in the PeP gene product, a manganese-dependent protein, to be suppressed [13].

In order to elucidate the mechanism for innate immunity mediated by the product of the *Nramp1* locus we need to establish where in the cell the protein is located and to be able to develop assays to measure transport activity. As a primary step towards undertaking these goals we have developed polyclonal rabbit serum using a recombinant glutathione S-transferase protein as a fusion with murine *Nramp1* (amino acids 514–548). The reactivity of this serum is reported here. We show that an affinity-purified fraction of the serum is immunoreactive against a single protein band of 65 kDa expressed within macrophage cell lines following stimulation with interferon- γ (IFN- γ) alone or in combination with lipopolysaccharide (LPS). Similar results were obtained from primary, bone marrow-derived macrophages from both resistant and susceptible mouse strains. This study also establishes that the *Nramp1* polypeptide is associated with a subcellular organelle displaying the structural and functional characteristics of a phagolysosome.

EXPERIMENTAL

Preparation of *Nramp1* expression constructs and antisera

Anti-*Nramp1* antibody was prepared against recombinant fusion protein using the pGEX1 expression vector [15] corresponding to amino acids 514–548 of the *Nramp1* sequence by routine PCR cloning techniques. Clones were selected for fusion-protein expression on mini-preparations (10 ml) of protein purified over glutathione–agarose by batch methods and analysed by SDS/PAGE and staining with Coomassie Blue. Recombinant clones were further characterized by DNA sequencing, with DNA sequence analyses performed using the GCG package provided by the U.K. Human Genome Mapping Project. Antiserum was raised against the fusion protein (0.5 mg per rabbit per injection) emulsified in Freund's complete or incomplete adjuvant in New Zealand White rabbits by intramuscular injection.

Cell culture

Cells were the well-characterized microglial cells N11 and N13 [16], or the human THP1 pre-promonocytes, or primary, bone marrow-derived macrophages, and were cultured as described previously [17] or as recommended by the European Collection of Animal Cell Cultures. Cells were plated either on to tissue-grade plates (microglial cells) or bacterial-grade plastic (bone marrow-derived macrophages) and treated with human or murine IFN- γ (50 units/ml) (Genentech) alone or with LPS (100 ng/ml). Protein concentrations of cell extracts were determined using the bicinchoninic acid protocol (Pierce). Routinely 20 μ g of total protein was loaded on a single track for SDS/PAGE. Immunoblotting was performed by standard procedures and detection achieved using horseradish-peroxidase-conjugated goat anti-rabbit secondary antibody. Blots were visualized by enhanced chemiluminescence (Amersham). Nitrite production by stimu-

lated macrophage lineage cells was quantified by using the Griess reagent [10].

Transient transfection into Cos-1 cells

To assess the specificity of the anti-*Nramp1* antisera, transient transfection into Cos-1 cells was performed using lipofectamine (BRL) according to the manufacturer's instructions with a pcDNA3 *Nramp1* (Gly¹⁶⁹) expression plasmid and a derivative containing a Large T epitope tag [18] at the C-terminal, prepared by standard PCR cloning techniques. Cells were harvested 48 h post-transfection and treated as above.

Affinity purification of the anti-*Nramp1* immunoreactive serum

Purification was performed by passage of the serum down an immobilized maltose-binding protein (MBP)p*Nramp1* Affi-gel 15 column (1 ml) as described [13]. MBPp*Nramp1* plasmid was prepared as above for pGEX*Nramp1*. The recombinant protein was affinity purified on amylose resin (NEB) as described by the manufacturers.

Immunostaining and confocal microscopy

Bone marrow-derived macrophages, prepared using purified recombinant CSF-1 from N20 B10.L-Lsh^r congenic mice carrying the resistant *Nramp1* allele on a B10 genetic background, were treated with 100 units/ml recombinant murine IFN- γ (Genzyme Diagnostics, West Malling, Kent, U.K.) and 50 ng/ml LPS (24 h), washed with PBS, fixed in 4% (w/v) paraformaldehyde in PBS, quenched with 100 mM glycine in PBS, and post-fixed with methanol. To detect *Nramp1*, cells were incubated in 10% (v/v) normal swine serum (Dako, High Wycombe, Bucks., U.K.) for 1 h, before addition of rabbit polyclonal antiserum or the affinity-purified antibody (1:100) for 2 h at room temperature. Control preparations were incubated with normal rabbit serum, or without any primary antibody layer. Biotinylated swine anti-rabbit IgG (1:300; Dako) was added for 45 min, followed by fluorescein-conjugated streptavidin (1:100; Dako) for 45 min. Antibodies and conjugate were diluted in 0.5% (v/v) Nonidet in PBS. Cells were washed 3 times in 0.05% Nonidet in PBS between each step. Nuclei were visualized by counterstaining with propidium iodide (5 ng/ml). Cells were washed and mounted in Vectashield (Vector Laboratories, Peterborough, U.K.), and viewed with a Nikon optiphot-2 epifluorescence microscope coupled to a Bio-Rad MRC1000 confocal laser scanning attachment (Bio-Rad, Hemel Hempstead, Herts., U.K.). The microscope settings were standardized throughout: iris 1.6 mm; gain 1500U; black level –2 for green fluorescence; iris 1.0 mm; gain 1100U; black level –2 for red fluorescence.

Preparation of phagolysosomal fractions

Phagosomes were isolated by magnetic selection [19] from cultures of N13 microglial cells [(1–5) $\times 10^6$], either resting or stimulated as above, and incubated for up to 2 h with Dynal 3 μ m diameter tosyl-activated polystyrene beads (Dynal catalogue No. 142:03) coupled to mouse IgG (Sigma I-5381).

RESULTS

Anti-*Nramp1* serum is reactive towards a 65 kDa protein in IFN- γ -stimulated macrophages

Antiserum to murine recombinant *Nramp1* was used to detect the *Nramp1* polypeptide by Western blotting using whole serum against extracts from murine macrophage and fibroblast cell

Figure 1 Macrophage-restricted expression of a 65 kDa protein reactive against anti-Nramp1 antisera

(a) Western blot of murine microglial cell (N11) extracts (20 μ g) (lanes 1 and 2) or from murine NIH3T3 cells (lanes 3 and 4) either resting (lanes 1 and 3) or stimulated for 24 h in the presence of IFN- γ (50 units/ml) and LPS (100 ng/ml) (lanes 2 and 4). Anti-serum was crude rabbit polyclonal serum raised against whole glutathione-S-transferase-Nramp1 (amino acids 514–548) recombinant fusion protein. (b) Direct repeat of (a), but using affinity-purified serum following positive selection of IgG after passage down Nramp1 (amino acids 514–548) MBP fusion protein. (c) Western blot showing response of Nramp1 synthesis in N11 cells (lanes 1–4) and NIH3T3 cells (lanes 5–8) following administration of control media (lanes 1 and 5); IFN- γ (lanes 2 and 6); LPS (lanes 3 and 7) and IFN- γ and LPS (lanes 4 and 8) for 24 h. Positions of 65 and 30 kDa proteins are shown on the left.

lines. Initially, reactivity was observed against several proteins, although polyclonal anti-(glutathione S-transferase) serum detected no proteins from these macrophage cell (results not shown). A single and discrete band of 65 kDa was observed in IFN- γ + LPS-stimulated macrophages, but not in fibroblasts or resting macrophages (Figure 1a), which is consistent with the *Nramp1* mRNA expression data [20]. The apparent molecular mass of this inducible band correlated well with the predicted size of the *Nramp1*-derived amino acid sequence (59.7 kDa), and immunoreactivity was associated with 100000 *g* particulate material rather than the post-microsomal supernatant (results not shown). Binding to and elution from an immobilized Nramp1 affinity resin allowed for the removal of the IgG molecules recognizing the protein bands common to both fibroblast and macrophage cell types (Figure 1b). By Western blotting this purified reagent was reactive exclusively towards the expected macrophage-expressed IFN- γ -inducible protein of 65 kDa and also to the Nramp1 MBP fusion protein, but not non-recombinant MBP (results not shown). In a series of experiments to identify the co-operativity of the agonists for Nramp1 protein expression, we analysed microglial cell extracts treated with IFN- γ and LPS alone and in combination. For the N11 macrophages we observed no effect on Nramp1 expression with LPS alone, but its effects

Figure 2 Expression of 65 kDa Nramp1 in primary bone-marrow-derived macrophages from Balb/c and CBA mice

(a) Western blot of Balb/c (lanes 1–4) and CBA (lanes 5–8) macrophage extracts following 24 h treatment with control media (lanes 1 and 5), IFN- γ (lanes 2 and 6), LPS (lanes 3 and 7) and IFN- γ and LPS (lanes 4 and 8). (b) As in (a) but using affinity-purified serum, specific for the 65 kDa Nramp1 band. Positions of 65 and 30 kDa proteins are shown on the left.

were synergistic with those of IFN- γ (Figure 1c). Bone-marrow macrophages prepared from Balb/c and CBA mice, representative strains for susceptible (D169) and resistant (G169) mice respectively (and confirmed by sequencing reverse-transcriptase PCR products prepared across the 169 codon; results not shown), both expressed 65 kDa Nramp1, but with subtle differences in the induction between strains, and also a marked effect of LPS on expression when administered alone (Figure 2a), compared with macrophage cell lines. The purified serum allowed detection of the 65 kDa band in the absence of the contaminating proteins common to other cell lineages (Figure 2b).

Heterogeneous polypeptides encoded by the *Nramp1* (NRAMP1) locus in different cell types

Previous studies employing N-terminal-reactive anti-Nramp1 antibodies have shown the *Nramp1* locus to encode a heavily glycosylated, disperse polypeptide of 90–100 kDa migrating with increased mobility (38 kDa) following treatment with PNGaseF [20]. Two experimental approaches described here provide evidence that the antiserum reactive towards the 65 kDa band in microglial cells is recognizing authentic Nramp1 protein and provides evidence for heterogeneity in the size of the Nramp1 polypeptide in cells. Firstly, transient transfection of monkey kidney Cos-1 cells using Nramp1 (Gly¹⁶⁹) expression plasmids containing either the full-length native sequence or a C-terminal fusion with the Large T epitope (PPEPET) was performed. Cell

Figure 3 Transient expression of immunoreactive Nrap1 in Cos-1 cells

Cos-1 cells transiently transfected with lipofectamine using pcDNA3 *Nrap1* Gly¹⁶⁹ expression plasmid (lane 3), C-terminal Large T antigen epitope-tagged *Nrap1* expression plasmid (lane 4) or mock transfected cells (lane 5). Extracts were prepared 48 h post-transfection and analysed by Western blotting (a) with anti-Nrap1 antiserum or (b) with KT3 mouse monoclonal antibody. Also shown on the same blot are samples from N11 microglial cells, resting (lane 1), or stimulated with IFN- γ and LPS (lane 2). Positions of 90, 65 and 40 kDa proteins are shown on the left.

extracts were prepared 48 h post-transfection for Western blotting (Figure 3) with either the 65 kDa Nrap1-reactive serum or a mouse monoclonal, KT3 [21], that recognizes the Large T antigen epitope tag. Neither antiserum showed any reactivity towards mock transfectants other than KT3 reacting with the endogenous 94-kDa Large T antigen. The Nrap1 antiserum reacted with the predicted 65 kDa band in activated microglial cells. In transfectant Cos-1 cells the major product was approx. 40 kDa and consisted of not a single band, but a ladder of closely spaced bands migrating with similar mobility to the PGNaseF-treated protein described previously [20]. For the epitope-tagged construct, a similar ladder was observed, but of a slightly reduced mobility resulting from the expressed protein carrying the tag. A band co-migrating with the authentic 65 kDa Nrap1 polypeptide was apparent in cells transfected with the non-tagged construct and faintly visible from the tagged transfectants. Higher-molecular-mass material was present in both transfectants and comparable with the 90–100 kDa species [20]. The KT3 serum only detected additional bands of approx. 40 kDa in the tagged construct transfectant cells, as predicted, but no band co-migrating with 65 kDa Nrap1 was observed as a result of the lower sensitivity of this reagent and the lower level of expressed protein resulting from either the reduced transfection efficiency or the stability of the hybrid, tagged protein. Secondly, the affinity-purified antiserum reacts with a single, discrete high-molecular-mass protein in cell extracts prepared from human THP1 pre-promonocytes following treatment with IFN- γ (Figure 4). The interaction with IFN- γ and LPS for the enhanced induction of human NRAMP1 in THP1 cells is similar to the requirements for the induction of the protein in murine cells (compare Figures 1 and 2 with Figure 4).

Figure 4 Expression of heterogeneous Nrap1 polypeptides in murine microglial N11 and human pre-promonocyte THP1 cells

Human THP1 (lanes 3–5) and N11 cells (lanes 1 and 2) were untreated (lanes 1 and 3) or treated with IFN- γ (50 units/ml) alone (lane 5) and with LPS (100 ng/ml) (lanes 2 and 4). Whole-cell extracts were prepared and analysed by Western blotting. Positions of 150 and 65 kDa proteins are shown on the left.

Iron chelation from microglial cells modulates Nrap1 polypeptide expression

Recent studies have shown that iron chelation/loading can influence the expression levels of *Nrap1* mRNA post-transcriptionally [21]. In order to provide more evidence for the 65 kDa protein being the product of the murine *Nrap1* locus, we investigated the effects of iron deprivation on Nrap1 polypeptide expression. Cultures of microglial cells were subjected to different iron levels by titration of the iron chelator deferoxamine (Figure 5). The response to deferoxamine treatment is influenced by the state of cellular activation. For cells only exposed to IFN- γ , deferoxamine (10, 100 μ M) causes an increase in the level of 65 kDa Nrap1 polypeptide expression (compare lane 1 with lanes 3 and 4). In contrast, cells treated with both IFN- γ and LPS respond to deferoxamine by diminishing the amount of Nrap1 expression at 1, 10 and 100 μ M. It is unlikely that the high concentrations of deferoxamine are cytotoxic, since for cells treated with IFN- γ alone, the higher concentrations

Figure 5 Modulation of Nrap1 polypeptide expression by iron chelation

N11 cells were pretreated with deferoxamine: 0 (lanes 1 and 5), 1 μ M (lanes 2 and 6), 10 μ M (lanes 3 and 7), 100 μ M (lanes 4 and 8), for 1 h and then supplemented with IFN- γ (10 units/ml; lanes 1–4) or LPS (100 ng/ml) + IFN- γ (10 units/ml; lanes 5–8). Cells were cultured for a further 24 h and extracts prepared for Western blotting. Samples of the cell culture supernatant were taken for nitrite determination by Griess: 107, 167, 120, 79 pmol/ μ g (lanes 1–4) and 133, 141, 110, 110 pmol/ μ g (lanes 5–8). Position of 65 kDa protein is shown on the left.

Figure 6 Time course for the appearance of 65 kDa *Nramp1*

N11 (a) and N13 (b) microglial cells were incubated with IFN- γ (50 units/ml) and LPS (100 ng/ml) for 0, 3, 6, 12, 18 and 24 h (lanes 1–6 respectively). Cell extracts were prepared and analysed by Western blotting for *Nramp1* expression. Culture media were harvested at similar time points and analysed by Griess for nitrite accumulation. Nitrite levels from N11 cultures corresponded respectively to 2.4, 1.4, 99, 211, 383, 580 pmol/ μ g of protein and from N13 cells to 3.2, 0.7, 10, 55, 177, 294 pmol/ μ g of protein for lanes 1–6. Position of 65 kDa protein is shown on the left.

1	1–	MTGD	KGPORLSGSS	<u>YGS</u>	ISSPTSP	TSPGPQQAPPE	–36
2	1–	MISD	KSPFRLSRPS	<u>YGS</u>	ISSLP	PAPQPAPCRE	–33
4	1–	MVLDPKE	KMPDDGASGDH	GDSASL	GAINPAYSNS	SLPHSTGDSE	–44
1	516–	WTCC	LAHGATFL	AHSSHHHFL	<u>YGLLE</u>	EDQKGE-TSG	–550
2	513–	WTCC	LAHGATFL	THSSHKHFL	<u>YGLPNE</u>	EQGGVQSG	–548
3		WQCL	IALGMSFL	DCGHTVS	ISKGLT	TEATRQYVK	
4	526–	WQCL	IALGLSFL	DCGRSYRL	GLTAQPELY	LLNTVDADSVSR	–568

Figure 7 Multiple *Nramp1* + 2-derived amino acid sequence alignment of C- and N-terminal cytoplasmic domains and identification of putative endocytic targeting motifs

Sequences were aligned using the Clustal multiple amino acid sequence alignment program over regions of human NRAMP1 (GenBank Database accession number L32185), mouse *Nramp1* (x75355), human NRAMP2 (J37347) and mouse *Nramp2* (J33415) corresponding to sequences 1, 2, 3 and 4 respectively. Amino acid residues conserved in all four sequences are shown in bold and tyrosine-based or dileucine motifs are underlined. Numbers correspond to the amino acid residues of the intact proteins, except for the sequence of human NRAMP2 which is incomplete at the N-terminal.

promoted induction of the *Nramp1* polypeptide. The changes in the level of *Nramp1* expression are not directly followed by changes in the level of nitrite detectable in the culture supernatants, unlike the initial appearance of *Nramp1* and nitrite (see below).

***Nramp1* protein is induced within 6 h after stimulation with IFN- γ and LPS**

The time for the appearance of *Nramp1* in microglial cells was examined, in order to determine optimal conditions for future

localization studies. *Nramp1* polypeptide appearance coincided with the detection of nitrite in the culture media, an index of *iNos* gene activity (Figure 6). *Nramp1* protein was detectable from as early as 6 h following cell stimulation in both cell lines, and accumulated in both N11 and N13 cells until 24 h. However, expression of *Nramp1* in N11 cells was greater at 12 h, before declining to lower levels than in N13 cells at later time points. This slight drop in *Nramp1* expression in N11 cells is likely to be the result of diminished cell viability due to either the acidification of the tissue culture media, or the accumulation of high levels of nitrite. It is also apparent that the magnitude of nitrite accumulation in N11 cells is significantly larger than in N13 cells, and over a number of determinations this has consistently been 2-fold at 24 h.

***Nramp1* + 2 sequence alignment highlights tyrosine-based and dileucine motifs for endocytic targeting**

Inspection of a clustal alignment of the four *Nramp1* + 2-derived amino acid sequences from human [14,22,23] and mouse [4,5,24] C-terminal domains reveals the presence of a conserved Y in *Nramp1* sequences within the context of surrounding sequences YGLL and YGLP for mouse and human respectively (Figure 7). Similarly, a second conserved Y is found within the N-terminal cytoplasmic domain for human and mouse *Nramp1* sequences within the context of YGSI for both. *Nramp2* sequences possess a dileucine motif within the carboxy region and this motif is also found embedded within the tyrosine-based motif for human NRAMP1. For resident, integral membrane proteins of the endocytic pathway, these two motifs have been defined within cytoplasmic, C-terminal domains and proposed as being functionally interchangeable [25–27] for appropriate endosomal targeting. The tyrosine-based motif has a requirement for the Y residue to be present in a motif YXXZ, where X corresponds to any residue and Z to a hydrophobic residue, consistent with the motifs identified in *Nramp1* sequences.

Macrophage protein of 65 kDa localizes to a subcellular membrane

Confocal microscopy using the anti-(C-terminal) antibody and the affinity-purified product confirmed our observation that affinity purification had enriched, for *Nramp1*, detection and staining of an intracellular vesicular compartment. At 1:100 (Figure 8), the original antiserum showed generalized staining to intracellular membranes, whereas the affinity-purified antibody stained only the membranes surrounding an intracellular vesicular compartment. The endosomal/lysosomal nature of this compartment was confirmed by our demonstration that the membranes surrounding leishmanial phagosomes are positive for *Nramp1* staining (results not shown).

***Nramp1* immunoreactive polypeptide of 65 kDa associates with a functional phagolysosomal vesicle**

Phagosomes from N13 microglial cells, either resting or following stimulation with IFN- γ alone or with LPS, and allowed to phagocytose IgG-coated magnetic beads, were prepared by magnetic selection. Equal protein from whole-cell extracts and phagosome extracts were analysed for *Nramp1* reactivity by Western blotting. The resulting blot (Figure 9) shows a substantial enrichment of the *Nramp1*, immunoreactive, 65 kDa protein associated with a functional phagosome fraction compared with whole-cell lysates from both IFN- γ - and IFN- γ + LPS-stimulated cells, indicating that the *Nramp1* polypeptide resides within functional phagosomal membranes.

Figure 8 Nrp1 localizes to an intracellular vesicular compartment

(A) Staining at 1:100 dilution of the original anti-(C-terminal) antiserum. (B) Staining with the affinity-purified antibody at 1:100. (C) Control with no primary antibody, the results for which were similar to those obtained with normal rabbit serum. Nuclei are stained with propidium iodide. Affinity purification has clearly enriched the membrane staining of the intracellular vesicular compartment. Bars = 10 μ m.

Figure 9 Immunoreactive 65 kDa Nrp1 is enriched in functional phagolysosomal vesicles

To identify the nature of the macrophage membrane harbouring the Nrp1 molecule, N13 microglial cells, resting (lanes 1 and 2), or treated with IFN- γ (lanes 3 and 4), or with IFN- γ and LPS (lanes 5 and 6), were selected as controls (lanes 1, 3 and 5), or used for phagocytosis of IgG-coated magnetic beads (lanes 2, 4 and 6). Phagosomes were prepared following cell lysis by magnetic selection (lanes 2, 4 and 6), and equal amounts of protein from phagosome and whole-cell extracts (lanes 1, 3 and 5) were analysed by Western blotting. Position of 65 kDa protein is shown on the left.

DISCUSSION

There has been a great deal of work directed towards understanding the molecular defect associated with the murine chromosome-1-disease susceptibility locus *Lsh/Ity/Bcg*, since its original identification in the late 1970s, and towards describing its influence over the fate of a number of apparently unrelated

pathogens (reviewed in [5a,28]). Despite all the genetic studies to map, isolate and definitively prove *Nrp1* candidacy as *Lsh/Ity/Bcg*, only very recently [9,20] has information appeared on the protein product itself, enabling hypotheses on its role in the macrophage cell to be tested. Outstanding questions that require addressing include the subcellular location of the protein within the macrophage and the nature of its transported substrate.

In this study we selected the C-terminal 35 amino acid domain for the preparation of immunoreactive serum, since this region of the Nrp1 molecule is hydrophilic and as such likely to be exposed in the native protein. The prepared immunoreactive serum detected a 65 kDa protein in a range of macrophage lineage cells, but not from cells not expressing the *Nrp1* mRNA. Several lines of evidence point to this polypeptide as being authentic Nrp1. Firstly, the cell-type specific expression characteristics of this protein are consistent with the finding of a PU.1 binding site in the vicinity of the transcription initiation site for several genes associated with macrophage expression, including the MCSF and interleukin-8 receptors, Fc γ R1b and *Nrp1* [30]. Secondly, the putative Nrp1, 65 kDa protein is regulated by LPS administration in primary macrophages and by LPS with IFN- γ in macrophage lines. Nuclear factor interleukin-6 (NF-IL-6) is proposed as the global regulator of LPS on gene transcription in cells, including macrophages. The *Nrp1* promoter region has been found to possess two consensus elements responsible for the binding of NF-IL-6 subse-

quent to transcriptional regulation [30]. Thirdly, the levels of the Nramp1, 65 kDa protein are exquisitely sensitive to the administration of IFN- γ , and a number of elements are found in the *Nramp1* promoter sequence that are consistent with the regulation of expression we have observed [30]. Finally, both the functional and confocal microscopy localization approaches used here are in agreement and consistent with the identified targeting motifs conserved in both human and mouse Nramp1 sequences. Definitive proof that the 65 kDa protein is Nramp1 awaits the outcome of direct, N-terminal protein sequence determination of the 65 kDa polypeptide product, although to date we have been unsuccessful in using this reagent for immunoprecipitation. These data strongly suggest that Nramp1 is located on an internal organelle membrane of an activated macrophage, and functionally associated with the phagocytic apparatus. The intimate contact Nramp1 is likely to display with an engulfed pathogen in a phagolysosomal vesicle will enable Nramp1 to exert a major effect on modulating the environment within that vesicle and the ensuing proliferative rate of the pathogen.

Recent reports [9,20] describe a band of approx. 90–100 kDa, reputed as being heavily glycosylated Nramp1, by immunoprecipitation from SDS and Triton X-100 extracts of transgenic thioglycollate-elicited macrophages harbouring a resistant copy of the transgene, or from cells expressing the endogenous resistant protein. The 90–100 kDa protein represents a major difference in size compared with the Nramp1 protein identified here. However, striking evidence that our affinity-purified antibody is seeing specifically the Nramp1 polypeptide sequence is shown by transfection studies in Cos-1 cells. In these cells the affinity-purified antiserum fails to react with any polypeptide species in mock transfectant or parental cells. Transient transfection of the *Nramp1* cDNA sequence into these cells promotes the synthesis of a range of polypeptide species that are reactive with the antiserum, which only detect a single and discrete protein band from extracts of activated macrophage cultures. In addition, the apparent size of some of the overexpressed Nramp1 polypeptide species in transiently transfected Cos-1 cells are directly comparable with the 90–100 kDa protein detected previously [9,20]. The reason for the range of polypeptide species identified in the Cos-1 cells is probably the overexpression and partial post-translational modification of synthesized proteins or unstable macrophage proteins expressed in cells of a non-macrophage lineage. The 150 kDa polypeptide identified in the human monocytic cell line in these studies also displays similar induction properties to the murine 65 kDa Nramp1 polypeptide described here. Other studies have suggested that human peripheral blood lymphocytes express a 60 kDa Nramp1 protein, which unlike our studies does not appear to be IFN- γ -inducible [31]. The size heterogeneity of the Nramp1 polypeptide between work described here and other studies [9,20] is possibly related to the variation in carbohydrate structure between Nramp1 from different macrophage populations, and other experiments have shown that more than 50 % of the size of the 90–100 kDa protein is carbohydrate in nature [20]. We have yet to show the extent, or whether the 65 kDa polypeptide is modified by glycosylation, although it is intriguing that the band of intermediate electrophoretic mobility, 50–60 kDa [20], was generated by lower concentrations of PNGaseF, thought to arise by removal of one of the two postulated, complex carbohydrate side chains. The addition of these structures may only occur completely *in vivo* by specific stimuli received by the murine macrophage, but not by cells activated under tissue-culture conditions. The potential implications of heterogeneity on the function of the Nramp1 polypeptide are of obvious great interest since not all macrophage cell populations express the *Nramp1* (*Lsh/Ity/Bcg*) phenotype.

A second discrepancy between this study and other reports [20] describing the Nramp1 polypeptide concerns the Asp¹⁶⁹ allele and whether such a protein is made. It has been clearly shown by applying gene targeting technology that this allelic form of *Nramp1* behaves in a manner that is indistinguishable from the null [8], but in this present study our experiments question whether the S allele may not be expressed. In Balb/c bone-marrow-derived cells, we observe the 65 kDa polypeptide and at similar levels to that from CBA mice (see Figure 2). This finding would suggest that the phenotype demonstrable *in vivo* is due to a malfunctioning Nramp1 D169 protein rather than to lack of expression. The two reports on the mouse protein [9,20] concluded that macrophages derived from susceptible mice (Nramp1 A169) do not express the 90–100 kDa polypeptide. Although bands of approx. 50, 55 and 110 kDa were clearly visible from immunoprecipitates of susceptible mouse macrophage extracts (see Figure 8) [20], these were excluded from being Nramp1, since the size (in particular the 110 kDa band) is incompatible with that of the open reading frame of *Nramp1* cDNA. It is possible that as a result of aberrant protein folding patterns the Asp¹⁶⁹ allele polypeptide is unavailable for immunoprecipitation. However, via Western blotting, SDS-denatured proteins from both mouse strains display epitopes that are reactive towards the C-terminal antibody.

The biochemical basis for the influence of the Nramp1 polypeptide on pathogen proliferation is still unclear. Sequence similarity with the SMF1 [14] gene product supports a high-affinity manganese (Mn²⁺) transport function [13] for Nramp1, but it is located on the endosomal membrane (the present study). Divalent cations like Mn²⁺ (and Fe²⁺) are known to play a major role in the survival of microbes within phagolysosomal vesicles, since many pathogenic organisms secrete defensive Mn- (and Fe)-dependent superoxide enzymes for scavenging reactive oxygen metabolites [32]. Also, iron depletion has been suggested as a mechanism by which the growth of intracellular pathogens may be regulated [33], and modulated expression of the 65 kDa Nramp1 polypeptide by iron chelation lends support for the association between iron metabolism and Nramp1 for an innate immune response. Nevertheless the effect of iron on Nramp1 polypeptide expression does not necessarily infer that Nramp1 operates by transporting iron, since iron chelation is likely to have many far-reaching effects within the cell. There are, however, data [34] suggesting that *in vivo* activated macrophages demonstrate differential Listericidal activity *in vitro*, depending upon the manner of activation. Furthermore, macrophages require sufficient iron for killing, but too much favours pathogen proliferation and prevention of Listericidal activity. The results in this present study showing a complex regulation of Nramp1 expression by iron chelation may provide a glimpse at a potential mechanism to explain these observations. Modulation in the level of expression of the 65 kDa Nramp1 polypeptide by iron chelation also provides extra evidence for the 65 kDa protein being authentic Nramp1, in view of the preliminary studies showing *Nramp1* mRNA regulation by influencing iron levels [21]. This finding, together with the extensive literature on the regulation of proteins associated with iron metabolism and uptake [35], will provide a base from which to elucidate the nature of the transport substrate for Nramp1. The ability to purify phagolysosomes from intact macrophage cells expressing either functional or null *Nramp1* alleles will allow these hypotheses to be tested on both the identification of the substrate and the direction of transport for the Nramp1 molecule. In addition, purified vesicles will enable experiments to be conducted to identify factors controlling transport, i.e. protein phosphorylation, and also to elucidate the energy requirements for transport. Elucidation of the molecular mechanism of this gene may be

helpful in the design of novel treatments for chronic infectious disease.

This work was supported by grants from the Royal Society and the Biotechnology and Biological Sciences Research Council of Great Britain (C.H.B.) and the Wellcome Trust (J.M.B.). We are indebted to Dr. Marco Righi for providing the N11/N13 microglial cells for use in these studies and to Drs. Mann and East for helpful discussions concerning these experiments.

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