Ectopic expression of *Nramp1* in COS-1 cells modulates iron accumulation

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Abstract ***Nramp1* (*natural resistance-associated macrophage protein*) controls innate immunity and encodes a transporter of unknown function. Here we describe an antibody to Nramp1 displaying immunoreactivity towards a mature heavily glycosylated polypeptide of 90–100 kDa and a precursor form of 45 kDa in macrophages. Ectopic expression of the *Nramp1* cDNA in COS-1 cells demonstrates that Nramp1 modulates cellular iron levels following loading with low molecular weight iron chelates. Surprisingly, Nramp1 does not enhance iron uptake, but expression is associated with reduced cellular iron loads. We propose Nramp1 may play a role in a salvage pathway of iron recycling.**

Key words: Nramp1 polypeptide; Macrophage; Transfection; Iron transport

1. Introduction

Natural resistance or susceptibility to infection toward a number of unrelated intracellular pathogens is controlled by the *Ity*/*Lsh*/*Bcg* locus on mouse chromosome 1 (see [1,2] for review). Experiments by Gros and colleagues, using mouse gene knockout [3] and transgenic [4] techniques, have confirmed that this phenotype is controlled by a single gene, namely *Nramp1* (*Natural resistance-associated macrophage protein*). Murine *Nramp1* encodes a polytopic integral membrane protein, with two consensus sites for N-linked glycosylation and an amino-terminal proline-rich sequence [5]. A sequence motif in one of the intracellular loops has been described as a consensus transport signature [6]. This latter observation, together with recent work on other members of the highly conserved *Nramp* gene family support the hypothesis that *Nramp1* encodes a transporter molecule. *Nramp2* (78% identity to *Nramp1* in mouse) transports a number of metal ions when expressed in frog oocytes [7]. Innate resistance or susceptibility to infection in mouse has been invariably associated with a single G169D amino acid substitution within transmembrane region 4 of the Nramp1 polypeptide sequence [8]. Such a change in a hydrophobic environment could be envisioned to have a deleterious effect on the putative transport function of *Nramp1*. Moreover, *Nramp1* gene knockout experiments demonstrated the $Nramp1^{D169}$ allele is functionally indistinguishable from the null mutant [3].

The Nramp1 polypeptide has been localised to intracellular vesicular membranes in resting cells, and co-purifies with bead containing phagosomes after challenge with IgG-coated latex beads [9,10]. Such a localisation suggested that Nramp1 could influence the micro-environment of the invading pathogen within the phagolysosome by either directing the efflux or influx of some essential substrate or toxin respectively. Recent data have established that the *Nramp*-related yeast genes, *SMF1* and *SMF2* encode high and low affinity manganese transporters respectively [11]. Supek and co-workers [11] proposed that Nramp1 may blunt a key defence mechanism of many micro-organisms by depleting the phagosomal lumen of Mn^{2+}. Depletion of the phagosome of divalent manganese would prevent the engulfed microbes from biosynthesising active defense enzymes such as manganese-dependent superoxide dismutase. It is likely that Nramp1 exhibits different transport properties to Nramp2, since the double SMF1/2 knockout yeast strain associated with hypersensitivity to EGTA and growth at alkaline pH could be complemented by *Nramp2* [12], but not *Nramp1*. Rodent Nramp2 transported not only Mn^{2+}, but a variety of other divalent metal cations, into *Nramp2* transfected *Xenopus* oocytes [7] including high affinity ferrous iron (Fe^{2+}) transport. This result is of particular relevance in light of a recent study where a candidate iron transporter gene, identified as *Nramp2*, was responsible for murine microcytic anaemia associated with a defect in intestinal iron uptake [13].

To investigate Nramp1 structure and function, we have generated an Nramp1-specific antiserum, and developed a model expression system for analysis of Nramp1 function. These findings demonstrate that the Nramp1 polypeptide exists in two forms dependent upon the level of protein glycosylation. Ectopic expression of the heavily glycosylated Nramp1 polypeptide in COS-1 cells modulated iron levels. This important finding has implications for how Nramp1 may confer resistance to microbial infection in vivo and the role of the protein in macrophage function.

2. Materials and methods

2.1. Preparation of Nramp1 expression constructs and antisera

Anti-Nramp1 antibody was prepared against recombinant fusion protein using the pGEX1 expression vector [14] corresponding to amino acids 1–82 of the *Nramp1* sequence by routine PCR cloning techniques. Recombinant clones were characterised by DNA sequencing. Antisera were 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.

2.2. Affinity purification of the anti-Nramp1 immunoreactive serum

Purification was performed by passage of the serum down an immobilised MBPp (maltose binding protein) Nramp1 Affi-gel 15 column (1 ml) as described [9]. MBPpNramp1 plasmid was prepared as above for pGEXNramp1. The recombinant protein was affinity purified on amylose resin (NEB) as described by the manufacturers.

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2.3. Cell culture and transient transfection

Cells used were the well characterised microglial cells N11 [15] and the COS-1 cell. N11 cells were plated onto tissue grade plates and treated with murine γ-IFN (10 units/ml) (Gentech) or with lipopolysaccharide (LPS, Sigma) (100 ng/ml) alone or in combination. Protein concentrations of cell extracts were determined using the BCA protocol (Pierce). Routinely 20 μg of total cell protein was loaded on a single track for SDS-PAGE. Immunoblotting was performed by standard procedures and detection was by HRP conjugated goat anti-rabbit secondary antibody. Blots were visualised by chemiluminescence (ECL, Amersham). Transient transfection into COS-1 cells was performed using lipofectamine (BRL) according to the manufacturer's instructions with a pcDNA3 *Nramp1*$^{gly-169}$ allele expression plasmid and a derivative containing a large T epitope tag at the C-terminus. Cells were harvested 48 h post-transfection as above for Western analysis or used in iron uptake experiments.

2.4. Treatment of N11 cell extracts with glycosidases

Extracts were prepared in 0.5% SDS containing β-mercaptoethanol (1%) either by boiling samples (20 μg) or by syringe lysis. Samples were treated with 1000 units PNGase F (BRL) according to the manufacturer's instructions and at room temperature overnight.

2.5. Iron uptake and ferrozine assay

To prepare the low molecular weight iron chelate Fe:NTA (nitrilotriacetate), $FeCl_3$ was dissolved in HCl and added to aqueous NTA in a 1:1 molar ratio. Transfected COS-1 cells were extensively washed in RPMI medium alone to remove serum and then incubated in RPMI medium containing 50 μM Fe:NTA for 4 h at 37°C. Plates were placed on ice, medium removed and the cells washed three times in ice-cold PBS, containing 5 mM EDTA (pH 7.4). Cell monolayers were harvested in PBS/5 mM EDTA and by scraping. Cells were transferred into a microfuge tube and washed a further two times in PBS alone. Aliquots were removed for protein determination (BCA, Pierce) and iron determination. Cellular iron levels were measured as described [16]. Briefly, washed cells were resuspended in distilled water, transferred to glass hydrolysis tubes to which an equal volume of HNO_3 (6 M) was added. Extracts were incubated for 18 h at 100°C to release total cell-associated iron. Iron levels were assayed using the ferrozine method [17], except that the ammonium acetate buffer was made to 15% (w/v) containing NaOH (3.2 M).

3. Results

3.1. Nramp1 reactive antiserum

To facilitate the study of Nramp1 an N-terminal reactive polyclonal antiserum was generated which was subjected to affinity purification, and designated B1-NT. The reactivity of B1-NT against the native Nramp1 polypeptide was confirmed by Western blotting of COS-1 cell extracts from cells transiently transfected with the plasmid pCDNA3 containing the full length *Nramp1* sequence (Fig. 1a and b). The *Nramp1* expression construct was engineered with a carboxy-terminal located large T epitope tag which is recognised by the monoclonal antibody KT3 [18]. Western blots of control extracts; and either mock transfectants or transfection with a pCDNA3/*iNos* (inducible nitric oxide synthase) construct, failed to demonstrate reactivity against B1-NT (Fig. 1a, lanes 1 and 3). Blotting COS-1 cells transfected with the epitope tagged *Nramp1* expression plasmid yielded B1-NT reactive bands clustered around 90–100 kDa and 45 kDa (Fig. 1a, lane 2). Probing an identical blot with the anti-large T antiserum KT3 revealed a similar pattern to B1-NT (Fig. 1b) and confirmed that B1-NT does cross-react with native Nramp1. The endogenous large T antigen in COS-1 cells, migrating at about 95 kDa in SDS-polyacrylamide gels, is recognised by KT3 (Fig. 1b).

Antiserum B1-NT was employed to examine endogenous *Nramp1* expression in the murine microglial cell line, N11

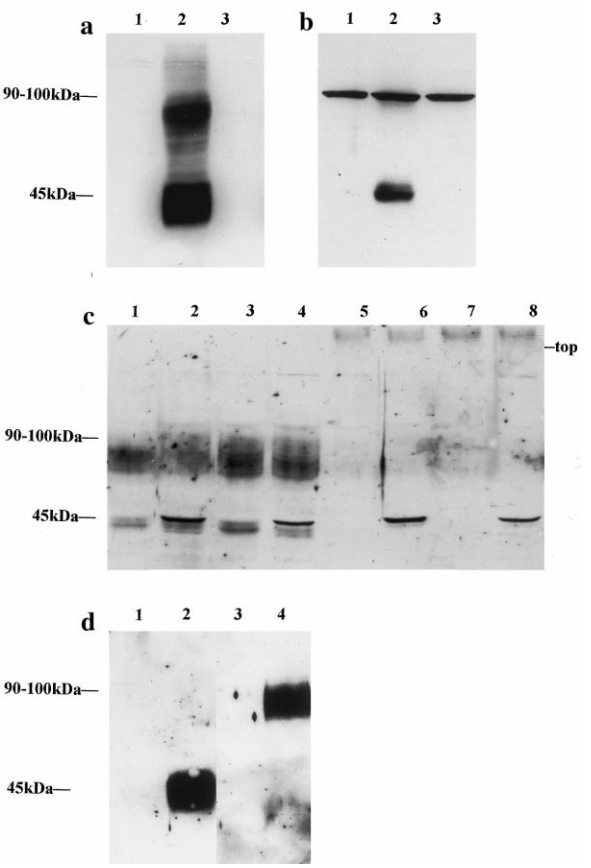

Fig. 1. Immune-detection of Nramp1 polypeptide in mammalian cells. a, b: Following transient expression of ectopic *Nramp1* cDNA in COS-1 cells. COS-1 cells were mock transfected (lane 1), transfected with pcDNA3 plasmid DNA incorporating the full length *Nramp1* cDNA (lane 2) corresponding to the G169 allele and a C-terminal located large T antigen epitope tag, or with pcDNA3 incorporating the murine *iNos* cDNA (lane 3) and using lipofectamine (BRL). 48 h post-transfection whole cell extracts were prepared and Nramp1 expression analysed from 20 μg samples using (a) the anti-N-terminal reactive Nramp1 B1-NT antisera or (b) the KT3 mouse monoclonal antibody and by Western blotting. Positions of 45 kDa and 90–100 kDa polypeptides are indicated of the left. c: Native Nramp1 polypeptide in N11 microglial, macrophage lineage cells. N11 (*Nramp1* G169 allele) cell extracts (20 μg) were analysed for anti-Nramp1 reactivity using the N-terminal antibody. Extracts were prepared in SDS-PAGE Laemmli sample by cell lysis using a gauge 23 syringe (lanes 1–4) or by boiling (lanes 5–8). Cells were untreated (lanes 1 and 5), treated overnight with γ-IFN (10 U/ml) (lanes 2 and 6), with lipopolysaccharide (100 ng/ml) (lanes 3 and 7) or with lipopolysaccharide and γ-IFN (lanes 4 and 8). Positions of 45 kDa and 90–100 kDa proteins are indicated of the left. d: 45 kDa is the precursor for the glycosylated 90–100 kDa Nramp1 protein. Whole cell extracts from untreated N11 cells were prepared in SDS and β-mercaptoethanol as above by boiling (lanes 1 and 3) or by syringe lysis (lanes 2 and 4). 20 μg aliquots of protein were incubated with PNGase F and analysed by Western blotting using the N-terminal anti-Nramp1 antisera. Samples were not reboiled subsequent to SDS-PAGE. Positions of 45 kDa and 90–100 kDa polypeptides are indicated on the left.

[9,15]. This cell line carries the resistant wild-type (Nramp1^{G169}) allele of *Nramp1* (Atkinson and Barton unpublished). *Nramp1* expression in these cells is constitutive, with little modulation upon cell stimulation with either γ-interferon (γ-IFN), lipopolysaccharide (LPS), or both. The general pattern of immunoreactivity, bands at 90–100 kDa and to a lesser

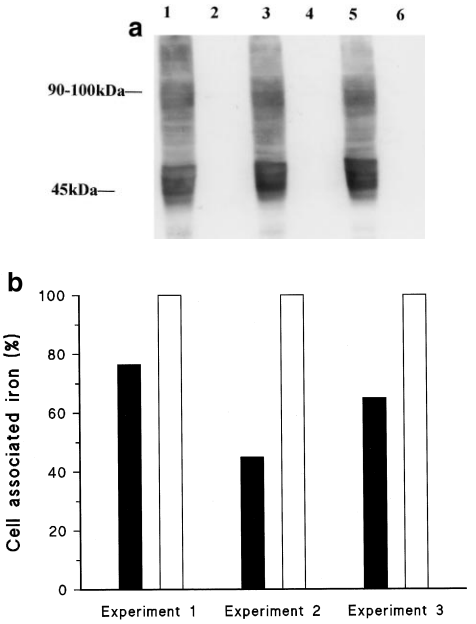

Fig. 2. Ectopic expression of Nramp1 cDNA modulates iron accumulation in COS-1 cells. COS-1 cells were transfected using the pCDNA3 plasmid incorporating (experimental) or lacking (control) *Nramp1* cDNA (G169 allele) as described in legend to Fig. 1. 48 h post-transfection cells were washed to remove serum and incubated in Fe^{3+}: nitrilotriacetate (50 μM) under serum-free conditions for a further 4 h. Cells were harvested, washed extensively in PBS/EDTA and samples prepared for confirmatory Western analysis or iron determination using the ferrozine reagent. a: Expression of Nramp1 in three independent iron accumulation experiments: experiments 1–3 in lanes 1 and 2; lanes 3 and 4 and lanes 5 and 6 respectively with vector alone controls (lanes 2, 4 and 6) or with Nramp1 cDNA (lanes 1, 3 and 5). Positions of 45 kDa and 90–100 kDa polypeptides are indicated of the left. b: Cellular iron levels associated with control (open bars) or Nramp1 transfectant cells (closed bars) from the samples as shown in (a). Iron levels for each control were arbitrarily set at 100%. Actual values (Nramp1/control) (nmol Fe/mg/4 h); Expt 1: 49/64; Expt 2: 17.4/38.5; Expt 3: 12.1/18.5.

extent 45 kDa, was similar to *Nramp1* transfected COS-1 cells (Fig. 1c, lanes 1–4). The fainter 45 kDa bands were not seen in every N11 Western blot; this variability between blots may well be the result of differences in the glycosylation status of Nramp1 (see below). In the above experiments, N11 extracts were prepared in Laemmli sample buffer prior to SDS-PAGE, but without the standard boiling procedure. Boiling of samples led to a loss of immunoreactive protein from the resolving gel (Fig. 1c, lanes 5–8). This was probably due to protein aggregation as high molecular weight material was often trapped in the sample wells. Similar findings have been reported by others [19]. Western blotting of N11 extracts with B1-NT antiserum revealed a γ-IFN induced 50 kDa protein that migrated more slowly than the Nramp1 45 kDa proteins (Fig. 1c, lanes 2 and 4). This protein was unaffected by heat treatment (Fig. 1c, lanes 6 and 8). The identity of this band is unclear, but it may be a product of the murine *Nramp2* gene as there is some sequence similarity between the two Nramp proteins over the first 82 residues. Expression of this polypeptide in fibroblast cells NIH 3T3 (Atkinson and Barton unpublished) excludes it from being derived from the *Nramp1* gene as the latter cells do not express the appropriate mRNA [6].

Glycosidase PNGase F treatment of N11 extracts demonstrated that the Nramp1 protein is heavily glycosylated in this cell line (Fig. 1d). Exhaustive PNGase F digestion caused the disappearance of the 90–100 kDa bands with the concomitant strong appearance of the 45 kDa immunoreactive bands (Fig. 1d, lanes 2 and 4). These results strongly suggest that the 90–100 kDa Nramp1 protein is the mature, fully glycosylated protein, whereas the 45 kDa polypeptides represent partially or non-glycosylated precursor forms. Samples that were boiled prior to glycosidase digestion failed to migrate into the resolving gel. This suggests that the boiling treatment either prevents access of the PNGase F to the appropriate glycosylation sites or that the aglycosyl form of the protein remains aggregated (Fig. 1d, lanes 1 and 3).

3.2. Nramp1 and iron levels in COS-1 cells

The transport activity of Nramp1 was investigated in the previously developed COS-1 cell Nramp1 transient transfection system. Iron delivery to cells was provided by complexing iron with the low molecular weight chelate, NTA, since cells readily take up iron in this form [20]. COS-1 cells were transfected as above. 48 h post-transfection, cells were incubated in 50 μM Fe^{3+}:NTA in the absence of serum. Iron uptake was for 4 h at 37°C prior to cell harvesting. In three independent experiments (Fig. 2), successful Nramp1 protein expression was confirmed by Western blotting with antiserum B1-NT (Fig. 2a). Similar Nramp1 protein expression, as determined by the degree of staining on the Western blot, was evident in all three experiments (Fig. 2a, lanes 1, 3 and 5). Extracts from cells transfected with vector alone failed to show any reactivity towards B1-NT (Fig. 2a, lanes 2, 4 and 6).

Iron incorporation using the ferrozine assay demonstrated a net accumulation of iron into the cells whilst COS-1 cells incubated in serum-free media alone, or in the presence of 50 μM NTA did not contain measurable cellular iron as assayed by this method (data not shown). In the presence of extracellular iron *Nramp1* transfected cells were associated with lower net iron levels when compared to the experimental controls. These reductions ranged from 24–55% and averaged 39% over the three experiments (Fig. 2b). Net iron accumulation over the 4 h time period was measured in nmol Fe mg^{-1} cell protein. There was some inter-experimental variation in iron levels in both control and Nramp1 positive cells (see legend to Fig. 2b), but the differences measured were consistent across experiments.

4. Discussion

The *Nramp1* locus has been attributed with many pleiotropic effects associated with macrophage antimicrobial activity [21–25], and the diversity of these effects has confused our understanding of this protein within the cell. Recently the *Nramp* gene family has been implicated with divalent cation transport and the intimate co-localisation of the Nramp1 polypeptide with the phagocytosed pathogen [9,10], has provided clues as to its putative function in antimicrobial activity.

In the present study anti-Nramp1 antiserum recognised exogenous Nramp1 polypeptides in transfected COS-1 cells, and native proteins in N11 microglial cells. Two immunoreactive proteins of 45 kDa and 90–100 kDa were identified and glycosidase treatment with PNGase F diminished immunoreactivity of the 90–100 kDa species and promoted the appearance of the 45 kDa species. PNGase F cleaves between the innermost *N*-acetylglucosamine and asparagine residues of

many N-linked oligosaccharide structures. The glycosylation is extensive given that the observed molecular weight of 90–100 kDa on SDS-polyacrylamide gels is some 30–40 kDa more than that predicted for Nramp1 based on its primary sequence. The abnormal mobility of the deglycosylated Nramp1 protein may arise from incomplete denaturation of the Nramp1 polypeptide, since boiling of macrophage extracts caused Nramp1 polypeptide aggregation with immunoreactivity detectable at the stack/resolving gel interface.

A similar pattern of Nramp1 glycosylation was observed from thioglycolate elicited peritoneal macrophages harvested from 129sv wild-type mice [19]. Our studies show that the extensive glycosylation of Nramp1 is not macrophage subtype specific, although heterogeneity has been reported in RAW264.7 cell transfectants ([10]; Atkinson and Barton, unpublished) and may represent a strain-specific effect. Such extensive modifications have been proposed to be required for correct targeting, and stabilisation of proteins in the hostile environment found in lysosomes [26].

Here we present evidence demonstrating that Nramp1 can modulate the accumulation of extracellular iron. Ectopic expression of wild-type *Nramp1* in COS-1 cells was associated with a reduction in the measured iron content, of nearly 40% over three experiments. This result is supported by the work of Zwilling and co-workers [27] who noted a 50% reduction in the cellular iron content in γ-IFN stimulated macrophages from Nramp1-resistant mice compared to macrophages from Nramp1-susceptible mice. It is unclear how Nramp1 modulates total cellular iron levels given that Nramp1 is localised to intracellular membranes and not the cell surface [11,12]. Nramp1 could be involved in the recycling of iron and one essential function of phagocytes is to degrade and recycle dead or dying cells including erythrocytes. By directing iron efflux Nramp1 may participate in the iron salvage pathway in the resting macrophage [28].

In the present study, iron loading was achieved in the absence of transferrin, by presenting iron to the cells complexed with NTA in serum-free media. Iron NTA is unique among non-physiological iron chelators in that it holds iron in a soluble form at neutral pH, whereas under mildly acidic conditions free iron salts form hydrolytic polymers from which the iron is relatively inaccessible. Iron in such low molecular weight chelates is readily taken up by cells [20] and is thought to be internalised via melanotransferrin (p97) found on the surface of many cell types [29]. Iron bound p97 is internalised by an undefined endocytic process where iron is released and p97 is recycled back to the cell surface [30]. The transport protein required to shuttle released iron from p97 (or transferrin) containing endocytic vesicles into the cytosol is currently unknown. However, based upon its endosomal location, it is tempting to speculate that Nramp1 fulfils this function in professional phagocytes. Therefore, it might be speculated that in Nramp1 transfected COS-1 cells, internalised iron was more efficiently transported in to the cytosol from the endosome than in Nramp1 minus controls. Increases in cytosolic redox active 'free' iron would be buffered by ferritin and other chelating agents, but in the short term the efflux of iron from the cell may be expected to increase. In the absence of Nramp1, the movement of iron from the endosome to the cytosol would be slowed, resulting in a build up of iron in the endosome and reducing iron efflux from the cell. These data form the basis for understanding the role of Nramp1 directed iron transport in cells and to address its role in antimicrobial activity.

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