The Nramp (Slc11) proteins regulate development, resistance to pathogenic bacteria and iron homeostasis in *Dictyostelium discoideum*

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Summary

The *Dictyostelium discoideum* genome harbors two genes encoding members of the Nramp superfamily, which is conserved from bacteria (MntH proteins) to humans (Slc11 proteins). Nramps are proton-driven metal ion transporters with a preference for iron and manganese. Acquisition of these metal cations is vital for all cells, as they act as redox cofactors and regulate key cellular processes, such as DNA synthesis, electron transport, energy metabolism and oxidative stress. *Dictyostelium* Nramp1 (Slc11a1), like its mammalian ortholog, mediates resistance to infection by invasive bacteria. We have extended the analysis to the *nramp*2 gene, by generating single and double *nramp1/nramp2* knockout mutants and cells expressing GFP fusion proteins. In contrast to Nramp1, which is recruited to phagosomes and macropinosomes, the Nramp2 protein is localized exclusively in the membrane of the contractile vacuole, a vesicular tubular network regulating cellular osmolarity. Both proteins colocalize with the V-H+-ATPase, which can provide the electrogenic force for vectorial transport. Like *nramp1*, *nramp2* gene disruption affects resistance to *Legionella pneumophila*. Disrupting both genes additionally leads to defects in development, with strong delay in cell aggregation, formation of large streams and the electrogenic force for vectorial transport. Single and double mutants display differential sensitivity to cell growth under conditions of iron overload or depletion. The data favor the hypothesis that Nramp1 and Nramp2, under control of the V-H+-ATPase, synergistically regulate iron homeostasis, with the contractile vacuole possibly acting as a store for metal cations.

Key words: *Dictyostelium discoideum*, Iron transporter, Nramp1, Nramp2, V-H+-ATPase, Iron homeostasis, Cell growth, Development, *Legionella pneumophila*, Bacterial infection, Phylogenesis

Introduction

The natural resistance-associated macrophage protein (Nramp) or solute carrier 11 (Slc11) family includes functionally related proteins defined by a conserved hydrophobic core of ten-twelve transmembrane domains. All members are proton-coupled transporters that facilitate absorption of divalent metal ions, including Fe²⁺, Mn²⁺, Zn²⁺, Co²⁺ and Cd²⁺ (Courville et al., 2006; Forbes and Gros, 2001; Nevo and Nelson, 2006). In mammals two homologs are found, Nramp1 (Slc11a1) and Nramp2 (Slc11a2 or DMT1). Nramp1 is found exclusively in macrophages and granulocytes, is localized in vesicles of the endosomal pathway and recruited to the phagosomal membrane upon phagocytosis (Canonne-Hergaux and Gros, 2004; Gruenheid et al., 1997). By controlling divalent metal concentrations, Nramp1 regulates the intracellular replication of pathogens. In mice, naturally occurring or experimentally induced mutations in Nramp1 cause susceptibility to infections by invasive bacteria. Mutations in humans may genetically predispose an individual to diseases, including tuberculosis, leishmaniasis and autoimmune diseases (Courville et al., 2006; Li et al., 2011; Malik et al., 2005; Nevo and Nelson, 2006; Taylor and Kelly, 2010; Velez et al., 2009; Wick, 2011). In contrast to Nramp1, Nramp2 is localized in the plasma membrane of cells in several tissues, particularly the apical membrane of intestinal and renal epithelial cells, and is the major transferrin-independent iron uptake system in mammals (Canonne-Hergaux et al., 1999; Courville et al., 2006; Theil, 2011). Alternative splicing gives rise to Nramp2 isoforms, which are mainly found in recycling endosomes, and are required in combination with Nramp1 for efficient iron recycling (Lamy-Yuk-Tseung and Gros, 2006; Soe-Lin et al., 2010). Mutations in Nramp2 are responsible for microcytic anemia and serum and hepatic overload (Iolascon and De Falco, 2009). Genetic studies in rodents have shown that Nramp2 is essential for survival and a critical threshold of the protein is necessary to protect against disorders of iron and manganese homeostasis (Fleming et al., 1997; Gunshin et al., 2005).

Nramp proteins are widely distributed in eukaryotes and prokaryotes (Courville et al., 2006). Based on sequence analysis Richer et al. (Richer et al., 2003) have distinguished prototypical Nramps, present in amoebae, yeasts, plants and invertebrates, from archetypical Nramps, which can be also found in these species but are the only homologs present in vertebrates, including mammalian Nramp1 and 2. In Gram-positive and Gram-negative bacteria, the Nramp superfamily is represented by the proindependent manganese transporter (MntH) family (Papp-Wallace and Maguire, 2006; Richer et al., 2003).

The genome of the social amoeba *Dictyostelium discoideum* harbours two *nramp* genes. The first gene product that was
characterized was called Nramp1, as it displayed all the properties of the mammalian ortholog (Peracino et al., 2006). Similarly to mammalian Nrp1, *Dictyostelium* Nramp1 was expressed in vesicles of the endolysosomal pathway, was recruited to phagosomes, and its inactivation affected susceptibility to infection by *Legionella* and *Mycobacterium*. The protein was found to be required for H\(^+\)-dependent iron export in isolated phagosomes (Peracino et al., 2006), favoring the notion of iron depletion from the phagosome, as originally suggested for mammalian Nrp1 (Forbes and Gros, 2001). Although Fe\(^{2+}\) in phagolysosomes can produce hydroxyl radicals via Fenton reaction, thus potentially killing bacteria, depletion of the iron pool from the phagosome deprives pathogenic bacteria of metal ions that are critical for their survival. Invasive bacteria, such as *Legionella pneumophila*, *Mycobacterium tuberculosis* or *Salmonella enterica* serovar Typhimurium are known to absorb large amounts of iron, while at the same time they are resistant to oxygen radicals (Henard and Vazquez-Torres, 2011; Johnson and Wessling-Resnick, 2012; Kortman et al., 2012; Moalem et al., 2004; Robey and Cianciotto, 2002; Slauch, 2011). Recently, we also showed that *L. pneumophila* hinders recruitment of the V-H\(^+\)-ATPase, but not Nrp1, in the *Legionella*-containing vacuole in a process apparently involving inhibition of phosphatidylinositol-3-phosphate formation. By avoiding colocalization of V-H\(^+\)-ATPase with Nrp1 in the same vesicle, *Legionella* neutralizes the proton-driven iron export via Nrp1, and may even turn the protein activity to its advantage (Peracino et al., 2010).

Nrp1 is one of a few genes that have been implicated in *Dictyostelium* innate immunity against pathogenic bacteria (Bozzaro and Eichinger, 2011; Chen et al., 2007; Cosson and Soldati, 2008; Sillo et al., 2008; Steinert, 2011). *Dictyostelium* cells are professional phagocytes that live in the forest soil as solitary amoebae and grow by grazing on different species of bacteria (Bozzaro et al., 2008; Kessin, 2001). Depending on bacteria as the obligate source of food, *Dictyostelium* can also be a natural host of pathogenic bacteria, and several pathogens that only occasionally infect humans, including *L. pneumophila*, *M. tuberculosis*, *Salmonella typhimurium*, *Neisseria meningitidis* or *Pseudomonas aeruginosa*, have been shown to be pathogenic also for *Dictyostelium* (Bozzaro and Eichinger, 2011; Colucci et al., 2008; Hagedorn et al., 2009; Lima et al., 2011; Sillo et al., 2011; Steinert, 2011). The solitary *Dictyostelium* amoebae enter a social phase of life upon depletion of the food source. Starvation induces a developmental process, whereby cells assemble into multicellular aggregates by chemotaxis, giving rise to elongated slugs, where cells undergo differentiation into prestalk and prespore cells. After a period of migration, the slugs culminate and form fruiting bodies, each consisting of a stalk of vacuolated dead cells, bearing at the top a ball of mature spores (Kessin, 2001). During development, cells do not ingest external nutrients and are bound to recycle intracellular material, mostly by autophagy and protein degradation, for acquiring the energy and synthesizing all the cellular components required for development and cell differentiation (Kessin, 2001; Otto et al., 2003).

In this paper, we have characterized the second *nramp* gene of *Dictyostelium*, thus called *nramp2*. The *nramp2* gene is maximally expressed during the aggregation phase of development. We show that the protein is exclusively localized in the contractile vacuole network, a tubular-vesicular network regulating osmolarity (Clarke et al., 2002; Clarke and Heuser, 1997; Heuser et al., 1993). By generating single and double *nramp1/nramp2* knockout mutants, we show that Nrp2, like Nrp1, is required for resistance to invasive bacteria, and that the double mutation leads additionally to a strong delay in aggregation and to formation of multi-tipped aggregates. Under conditions of iron overload or depletion, growth of single or double KO mutants is differentially affected, suggesting that the Nrp proteins regulate synergistically iron homeostasis.

**Results**

**Characterization and developmental expression of the *nramp2* gene**

The *Dictyostelium* genome contains two genes, both located in chromosome 2, with evident sequence homology to genes of the Nrp1 superfamily. The *nramp1* gene (DDB_G0276973) is the ortholog of mammalian *nramp1* and was characterized previously (Peracino et al., 2006; Peracino et al., 2010). The *nramp2* gene (DDB_G0275815) encodes a putative integral membrane protein with 12 transmembrane domains and a M\(_{r}\) of 70 kDa, 10 kDa larger than Nrp1 (Fig. 1). The DNA sequence is intransonless, in contrast to *nramp1*, which harbours an intron at position 199–309. The protein sequence shares 38% identity and 75% similarity with Nrp1, and the similarity, as expected, is low in the N- and C-terminal outer membrane sequences (Fig. 1). Blast analysis against reference proteins placed Nrp2 within the Nrp superfamily (Fig. 2). It is phylogenetically closer to \(\alpha\)-proteobacteria MntH and to Nrp proteins from yeast, fungi and prokaryotes than to the Slc11 family, to which instead Nrp1 belongs (Fig. 2) (Richer et al., 2003). We extended this analysis to include all Nrp1 and Nrp2 homologs encoded in sequenced genomes of *Dictyosteliaceae*. As shown in Fig. 2, Nrp1 and Nrp2 homologs from *D. discoideum*, *D. purpureum*, *D. fasciculatum*, *D. lacteum* and *Pseudomonas pallidum* cluster in two relatively distinct groups. This is in sharp contrast with other lower eukaryotes, such as *Chlamydomonas rheinardii*, and *Saccharomyces cerevisiae*, which harbor respectively 2 and 3 Nramps, forming in both cases, however, a single group (Fig. 2). *Arabidopsis thaliana* possesses 6 Nramps clustering in two groups, both of which are more closely related to *Dictyostelium* Nrp1 than Nrp2.

Whereas *nramp1* gene expression is higher during growth and declines upon starvation remaining at low level during development (Fig. 3) (Peracino et al., 2006), *nramp2* gene expression was very low during growth, increased during the first 6 hours of development and declined after formation of tight aggregates (Fig. 3). This pattern is similar to that revealed by RNA-seq analysis, available from the *Dictyostelium* webpage (www.dictybase.org).

**Nrp2 is a resident membrane protein of the contractile vacuole**

To determine the cellular localization of Nrp2 we cloned the gene and generated a chimeric protein with green fluorescence protein (GFP) fused at the C-terminus. Nrp2(G)-GFP homogeneously labeled the membrane of the contractile vacuole (CV) network (Fig. 4A). In living cells plated on glass, the CV network, made of branched vesicles and tubules, is typically located near the ventral surface of the cell, and extends over a large area close to the plasma membrane, as can be better appreciated in a 3D reconstruction (supplementary material...
Movie 1). In higher confocal sections, no labeling was observed, except for peripheral vesicles of the CV adjacent to the plasma membrane (Fig. 4A). These results suggest that Nramp2 is a selective marker of the contractile vacuole. For comparison, cells expressing GFP-fused Nramp2 or Nramp1 and stained with antibodies against the vatA subunit of the V-H+-ATPase are shown (Fig. 4C). The V-H+-ATPase is a major component of the CV membrane, besides being also transiently recruited to endolysosomes (Bracco et al., 1997; Clarke et al., 2002; Gerisch et al., 2002; Peracino et al., 2006), whereas Nramp1 is not expressed in the CV, but in the Golgi, in tiny vesicles trafficking between Golgi and plasma membrane, and transiently in endo- and phagosomes (Peracino et al., 2006). Accordingly, Nramp1 colocalized with VatA in some vesicles in upper sections, but not in the lower CV (Fig. 4B), whereas there was full colocalization of VatA and Nramp2 in the CV, not however in VatA-positive vesicles in the upper sections (Fig. 4B).

The plasma membrane was usually devoid of Nramp2-GFP, although a clearly labeled rim all over the membrane was observed in some cells (Fig. 4C). This is very likely an artifact due to overexpression of the GFP-fused protein or to the cell adhering strongly to the substratum, as it was observed only in a minority of such cells. To further exclude in any case the possibility that Nramp2 is a plasma membrane protein, we incubated cells with TRITC-dextran for 5 min, washed the cells and followed macropinosome formation, with particular attention to cells expressing Nramp2-GFP in the plasma membrane. When macropinocytic cups and vesicles are formed, most membrane proteins are entrapped, at least transiently, in the endocytosed membrane. We never observed Nramp2-GFP decorating TRITC-filled macropinosomes, whether or not there was any Nramp2-GFP labeling of the plasma membrane (Fig. 4C,D). This is in sharp contrast with Nramp1 or the VatA subunit of the vacuolar ATPase, which were transiently recruited from internal stores to TRITC-containing vesicles shortly after uptake (Fig. 4C,D) (Clarke et al., 2002; Clarke et al., 2010; Peracino et al., 2006). Cells were also incubated with neutral red, which stains acidic vesicles. As shown in Fig. 4E, no Nramp2-GFP decorated vesicles turned red, in contrast to Nramp1-GFP. Thus we conclude that Nramp2 expression is confined to the contractile vacuole, and the protein is in any case excluded from the endolysosomal pathway.

Differential effects of single and double nramp gene disruption on development

We generated single nramp2 and double nramp1/2 KO mutants by using the Cre-loxP system (Kimmel and Faix, 2006) as detailed in supplementary material Fig. S1. The mutants were tested for growth in axenic medium and on N-agar with Escherichia coli B/r or for development on phosphate agar. Growth was not affected. All mutants were able to develop and form fruiting bodies, but developmental timing and fruiting body morphology were altered, particularly in the double mutant. Compared to the parental AX2 strain, the Nramp1 KO (HSB60) mutant (Peracino et al., 2006) started to aggregate at about the same time, but completed aggregation and formed tip mounds about 3 hours earlier, whereas the timing of postaggregative development was unaltered (Fig. 5). The Nramp2 KO mutant started aggregation and formed tip mounds with a 2 hour delay, but postaggregative development occurred with about the same timing as the parental strain. The double knockout mutant displayed a 6 hour delay in the beginning of aggregation till tip mound formation. Postaggregative development was also slowed down but to a lesser extent. In contrast to all other strains, the
formed larger aggregating streams on phosphate agar, which led to formation of large multitippered aggregates (Fig. 5A, B). Despite aggregate fragmentation due to multiple tip formation, the density of fruiting bodies was markedly reduced compared to the parental strain, and the fruiting bodies were on the average taller, though the proportion between sori and stalks was maintained (Fig. 5C, D). Expressing either Nramp1 or Nramp2-GFP in the double mutant led to rescue of both developmental timing and aggregate morphology compared to the single mutants expressing either Nramp1- or Nramp2-GFP, respectively.

Differential effects of iron depletion or overload on mutant growth in minimal medium

The Nramp proteins are proton-driven divalent metal transporters, with a preference for iron and manganese. We have shown this to apply also for Dictyostelium Nramp1 (Peracino et al., 2006), and we assume that Nramp2 also displays this function, based on the conserved protein sequence and general protein structure. As a first step to assess potential effects of nramp1 or nramp2 gene disruption on iron homeostasis, we tested the effects of iron on cell growth, by assaying growth in a minimal FM medium, containing aminoacids, vitamins, glucose and essential trace elements, previously shown to support Dictyostelium cell growth (Franke and Kessin, 1977). The FM medium contained 0.1 mM FeCl₃. We used the same recipe, but either omitted iron or added 0.01, 0.1 or 0.2 mM FeCl₃. AX2 or mutant cells exponentially growing in AX2 medium were diluted 100-fold in minimal medium to an initial concentration of 3–5 × 10⁴ cells per ml, and growth was assessed under shaking over a period of 2 to 4 weeks. Despite variability in the absolute values from experiment to experiment, the trend was similar. In culture medium without added iron, only the parental AX2 cells displayed steady though slow growth, with a doubling time of about 35–40 hours, reaching a density of 1 × 10⁶ cells per ml after 168 ± 24 hours (Fig. 6). The AX2 cells underwent on the average five to six duplications before reaching a plateau. The single as well as the double nramp1/nramp2 KO mutants grew only up to 1–2 × 10⁵ cells per ml during the first 72 to 96 hours incubation (doubling time: 36 to 45 hours), but then stopped growing (Fig. 6). Minimal medium supplemented with 0.01 (not shown) or 0.1 mM iron (Fig. 6) supported growth of AX2, single and double-null mutants, with all strains reaching a
concentration of $1 \times 10^7$ cells per ml after 168 to 196 hours incubation. At a concentration of 0.2 mM iron, growth was inhibited in all strains, more strongly in the parental strain, but remarkably less so in the double null mutant, which was the only one able to steadily grow to $3 \times 10^6$ cells per ml after 168 hours of incubation, with an average doubling time of about 26 hours (Fig. 6). After two doublings in the first 48 hours, the Nramp2 KO mutant strain took a further 120 hours to undergo a third doubling. The Nramp1 mutant and the parental AX2 strain doubled once in the first 24 hours and then needed about 100 and 140 hours, respectively, to double a second time. Thus it appears that 0.2 mM iron is somewhat toxic, particularly for wild type cells, whereas the double null mutant is relatively resistant. In contrast, all three mutants are much more sensitive to iron shortage than the parental strain. Cells transferred from axenic to the minimal medium contain iron released from digestion of peptone and yeast extract, and traces of iron are possibly available in the minimal medium even without added iron, thus it is not surprising that cells may undergo some duplication rounds. The results suggest nevertheless that inactivating either nramp1
or \textit{nramp2} renders the cells more sensitive than wild type cells to conditions of iron depletion. In contrast, inactivating both genes results in increased resistance to iron overload.

\textbf{Nramp2, like Nramp1, is required for resistance to \textit{L. pneumophila} infection}

We showed previously that Nramp1 KO mutants were more susceptible to infection by \textit{L. pneumophila} and \textit{Mycobacterium avium} (Peracino et al., 2006; Peracino et al., 2010). This prompted us to test whether disrupting Nramp2 also affected resistance to invasive bacteria and whether the double mutation resulted in any additional effect. Cells were incubated with \textit{L. pneumophila} and the rate of bacterial growth was assessed by the CFU (Colony Forming Unit) method. Despite high variation in the absolute values from experiment to experiment, the trend was similar in four different tests, as shown in the representative experiment in Fig. 7. The bacteria proliferated as well in the single \textit{nramp2}- as in the \textit{nramp1}-null mutant, and significantly better than in the parental AX2 strain. The double mutant behaved similarly to the single mutants up to 72 hours of infection, thereafter bacterial proliferation consistently declined. Visual examination showed, however, that at 96 hours there were clearly more extracellular bacteria in the medium containing cells of the double mutant than the single mutants, yet this finding escaped CFU detection. Since CFU measures viable bacteria, and the culture medium used for the infection assay does not support bacterial proliferation (Fig. 7) (Hägele et al., 2000; Peracino et al., 2006), these results are consistent with the hypothesis that between 72 and 96 hours of infection a higher number of cells undergo lysis in the double knockout than in the single mutants, releasing more bacteria into the medium. Because the released bacteria die in the assay medium, their higher number is not reflected in the subsequent CFU counts. Attempts to measure bacterial proliferation using GFP-labeled bacteria in fluorescence-activated flow cytometry failed, as GFP expression diminished drastically after 48 h of incubation.

Fig. 7. Effects of single or double gene disruption on development.

Starving cells of parental strain and mutants were plated on non-nutrient phosphate agar and development was monitored over time in a stereomicroscope. \textbf{Top panel:} the time (in hours) needed for beginning of stream formation (aggregation) or appearance of tipped aggregates, first fingers, pre-culminants and mature fruiting bodies is indicated for each mutant. (Mean of two experiments. The standard deviation was of \pm 1 hour or 2 hours for aggregation or the other developmental stages, respectively.) \textbf{Bottom panels:} developmental phenotype of the double mutant. (A) Cells aggregating after 12 hours starvation formed very large streams and (B) multi-tipped aggregates. (C) On the average taller fruiting bodies, but of reduced number, were formed, compared with (D) AX2 parental strain. Bars: 1 mm.
Iron is vital for all cells, as it modulates the cellular redox potential and regulates key cellular processes, from electron and oxygen transport to energy metabolism and DNA synthesis (Courville et al., 2006; Czachorowski et al., 2009). Metal cation transport is conserved from prokaryotes to higher eukaryotes (Courville et al., 2006; Hentze et al., 2010; Kakhlon and Cabantchick, 2002; Schaible et al., 2004). Despite the phylogenetic conservation of the Nramp proteins, D. discoideum is unusual in that it contains to distant Nramp proteins, Nramp1 in the Scl1 family, and Nramp2 which is more closely related to yeast SMF, C. rheinardtii Nramp and to α-proteobacteria MntH proteins. We have shown this to be true for all five Dictystelid species whose genomes have been sequenced. This is in sharp contrast with other lower eukaryotes harbouring more than one Nramp paralogs, such as C. rheinardtii, S. cerevisiae or A. thaliana, whose Nramp proteins form in each case a single cluster or, in the case of A. thaliana, two different, but very closely related clusters. It has been suggested that Nramp proteins originated in prokaryotes and were transferred endosymbiotically to the eukaryotic nucleus. Horizontal gene transfer from eukaryotes to prokaryotes has also been hypothesized to justify unexpected sequence diversity in MntH phyletic groups (Richer et al., 2003), and could explain the surprisingly closer similarity of Dictystelid Nramp2 to α-proteobacterial homologs than to other bacterial MntH sequences. If the Scl1 Nramp family derived from a duplication from an ancient Nramp, as is likely (Richer et al., 2003), then Dictystelium offers a unique opportunity to study the evolutionary divergence of these two types of Nramp proteins coexisting in a single organism.

The cellular localization of the Nramp1 and Nramp2 is very different. Like mammalian Nramp1, Dictystelium Nramp1 shuttles from the Golgi to the endolysosomal pathway, being recruited to phagosomes and macropinosomes shortly after their closure and recruitment of the V-H+ -ATPase. Colocalization of V-H+ -ATPase and Nramp1 in the membrane generates the right conditions for proton-driven transport of iron and other metals from such vesicles to the cytosol, exactly as occurs for vertebrate Nramp1 (COURVILLE et al., 2006; PERACINO et al., 2006). Nramp2 is instead expressed selectively in the membrane of the contractile vacuole network, which regulates osmolarity. As soil amoebae living in the forest Dictystelium cells are exposed to sudden changes of osmotic conditions, so that water regulation is critical for their survival. The contractile bladder and tubular network fills with water and swells under hypoosmotic conditions, giving rise to larger vacuoles that fuse with the plasma membrane to discharge excess fluid. Under hyperosmotic conditions, the cisternae flatten and the tubules get narrow (CLARKE and HEUSER, 1997; GERISCH et al., 2002; HEUSER et al., 1993). With a functioning CV, cells submerged in water survive, acquire the typical amoeboid shape and can also undergo development, whereas they rapidly round up and lyse if the CV cannot fulfill its function (DU et al., 2008; GERALD et al., 2002; MYRE et al., 2011).

The CV membrane is studded with vacuolar H+ -ATPase, which pumps protons into the lumen. Cells possess also a cytoplasmic carbonic anhydrase and a bicarbonate transporter in the CV membrane (Giggione and Gross, 1995; HEUSER et al., 1993). It has been suggested that production of bicarbonate from CO2 by the carbonic anhydrase and cotransport of the anion with protons into the lumen of the CV generate osmotically active carbonic acid that could draw water into the vacuole (CLARKE and HEUSER, 1997; HEUSER et al., 1993).

The finding that V-H+ -ATPase and Nramp2 co-localize in the CV membrane is suggestive of a potential role of the contractile vacuole in homeostasis of iron, and possibly other divalent metals. In the wild the major source of iron for Dictystelium cells is very likely the engulfed bacteria. We propose that iron, released after phagocytosis and digestion of bacteria (or ingested with peptone and yeast extract by macropinocytosis, in the case of laboratory axenic strains) is reduced to Fe2+ in phagosome or macropinososomes and transported via Nramp1 to the cytosol, where it can be used for cellular functions. Excess free iron could be transported in the CV that could thus function as a store or sink for the labile iron pool. The colocalization of Nramp1 or Nramp2 with the V-H+ -ATPase in phagosomes or the contractile vacuole, respectively, could explain vectorial transport by a common mechanism, namely electrogenic potential driven by the proton pump (Fig. 8). It is established for mammalian Nramp1 and Nramp2 (COURVILLE et al., 2006), Dictystelium Nramp1 (PERACINO et al., 2006), and for bacterial and yeast homologs of Dictystelium Nramp2 (COURVILLE et al., 2006), that the proton gradient generated by the vacuolar ATPase favors iron export from vesicles, such as phagosomes or yeast vacuoles, via Nramp. In the case of Dictystelium Nramp2, this mechanism of action would imply iron efflux from the contractile vacuole into the cytosol (Fig. 8). One objection to this mechanism is that the pH in the CV lumen is neutral, possibly due to proton buffering by cotransported bicarbonate (CLARKE and HEUSER, 1997; Giggione and Gross, 1995). It is possible however that a slight ΔpH across the CV membrane is present, which could be sufficient for iron efflux via Nramp2. In this context it is worth mentioning that a P-type Ca2+ ATPase (PAT1) is also localized to the CV and is involved in V-H+ -ATPase-dependent Ca2+ sequestration (MONIAKIS et al., 1999), as well as efflux to the cytoplasm, although it is unclear how the latter is regulated.
Fig. 8. Model for the function of Nramp1 and Nramp2 and of the contractile vacuole (CV) in iron homeostasis. Nramp1 is recruited to phagosomes after bacterial uptake and recruitment of the V-H\(^{+}\)-ATPase to the phagosomes, whereas Nramp2 and the V-H\(^{+}\)-ATPase are resident proteins of the CV network. Acidification by the V-H\(^{+}\)-ATPase leads to a proton gradient in the phagosome that favors cotransport to the cytosol of ferrous ions deriving from bacterial degradation (or digestion of peptone and yeast extract in case of growth in axenic medium). Iron is required for cellular functions, particularly in mitochondria. We suggest that the contractile vacuole act as a store/sink for labile iron, with sporadic or induced CV fusion with the plasma membrane leading to iron excretion. Iron can be transported across the CV membrane via Nramp2, and the directionality of this transport is regulated by the V-H\(^{+}\)-ATPase, which can provide the electrogenic potential across the membrane for iron influx into the cytosol, similar to what occurs in the phagosome. It is worth mentioning however that the pH in the CV lumen is mostly neutral (Giglione and Gross, 1995), therefore it is open to what extent this mechanism is constitutively active or efficient.

(Malchow et al., 2006). In any case a role for Nramp2 in mobilizing iron from the contractile vacuole is suggested by the iron-limited growth as well as the developmental delay of the nramp2-null mutant. If the model in Fig. 8 is correct, it can be speculated that Dictyostelium Nramp2 plays a role similar to the Nramp homologs in yeast (SMF3) or A. thaliana (AtNramp3 and AtNramp4), which mobilize iron from their respective vacuoles (Portnoy et al., 2000; Lanquar et al., 2005).

We have shown that disrupting nramp1, nramp2 or both has pleiotropic effects on development, cell growth under iron overload or shortage and on resistance to L. pneumophila. The developmental phenotypes are more difficult to correlate with specific defects in iron homeostasis, as very little is known in this regard in Dictyostelium. Development occurs in a simple salt solution made of Na- and K-phosphate, and can also occur in water. Exogenous iron or other metals are not essential, thus iron that is certainly required for mitochondrial activity and other iron-linked enzymatic activities is all fuelled by endogenous store. It is of interest that Nramp1 gene expression is downregulated during development, whereas Nramp2 expression is upregulated during the first 6 hours of development, coincident with the aggregation stage, and downregulated after formation of tight aggregates. Consistent with this regulation, Nramp1 gene disruption has little effect on development, resulting only in a slight acceleration of development, whereas inactivating Nramp2 delays the beginning of aggregation by 2 hours. Inactivating both genes results in a much stronger delay in cell aggregation, in large streaming aggregates and multitipped mounds. Despite fragmentation following multtip formation, fewer but on average taller fruiting bodies are formed in the double mutant compared to the parental strain. This phenotype can be explained by assuming that development of the aggregation competence is inhibited in the double mutant, such that fewer cells in the population are able to initiate autonomous cell-cell signaling that is required for chemotaxis and aggregation. It can be speculated that altered iron homeostasis affects mitochondrial activity which is necessary for proceeding in development. It is worth mentioning that starvation induces upregulation of mitochondrial enzymes, such as the citrate synthase. In addition, many Krebs cycle as well as oxidative phosphorylation enzymes and the iron-sensing aconitate hydratase are strongly upregulated during stem formation and aggregation up to the mound stage (Czarna et al., 2010). Thus, reduced mitochondrial efficiency, as a consequence of defective iron storage during growth and/or mobilization during development, could explain the delay of the single Nramp2 mutant and the developmental phenotype of the double mutant. It is suggestive that large streams and multitipped aggregates are also a common phenotype of autophagy mutants (Otto et al., 2003; Tung et al., 2010). Defective autophagy could also result in altered oxidative phosphorylation, as autophagy is required for recycling of intracellular components during Dictyostelium development (Otto et al., 2003).

The major effect of nramp1 inactivation is increased susceptibility to infection by L. pneumophila and M. avium (Peracino et al., 2006), resembling in this regard the effects of mutated mammalian Nramp1 (Cellier et al., 2007). Inactivating nramp2 or both genes also leads to increased sensitivity to Legionella infection compared to the parental strain, with the double mutant being very likely more affected, although this was not reflected in increased bacterial proliferation when measured by the CFU method. As mentioned in the results, this may be due to the CFU assay measuring bacteria that were viable at the time of the assay and not bacteria that had been released prior to the assay but subsequently died in the inhospitable culture medium. Thus it appears that any alteration of iron homeostasis via Nramp1 or Nramp2 disruption facilitates intracellular growth of Legionella. Whether this holds true also for M. avium is currently under study. In the absence of Nramp1, iron export from phagosomes or macropinosomes is strongly reduced (Peracino et al., 2006), thus it is conceivable that disruption of the nramp1 gene will lead to increased iron accumulation in the Legionella-containing macrophosome, a condition favoring Legionella growth (Robey and Cianciotto, 2002). How can inactivation of Nramp2, which is localized in the contractile vacuole, have a similar effect, if Nramp1 is active? We have recently shown that Legionella effectively hinders recruitment of the V-H\(^{+}\)-ATPase, but not Nramp1, to the Legionella-containing macrophosome (Peracino et al., 2010). In the absence of the vacuolar ATPase, Nramp1 pumping activity is neutralized, and iron may be sequestered by the Legionella (Peracino et al., 2006). Under these conditions, if Nramp2 is not functioning it is possible that cytosolic iron deficiency may affect basic cell functions, favoring Legionella growth.

In contrast to pathogens, non-pathogenic bacteria, such as E. coli, are taken up and digested at about the same rate by the mutants as well as the parental strain. Cell growth in either axenic medium, presumably containing about 0.05 mM Fe (Franke and Kessin, 1977), or minimal medium supplemented with 0.01 to...
0.1 mM iron chloride, is also not dramatically affected by \textit{nramp1} or \textit{nramp2} gene inactivation. In contrast, the single and double mutants are much more sensitive than the wild type strain to iron depletion and less sensitive, particularly the double mutant, to iron overload. Both results are consistent with the assumption that inactivating Nramp1, Nramp2 or both leads to lower accumulation of cytosolic iron, decreasing iron availability for cellular functions. As a consequence, when shifted from axenic medium to conditions of iron shortage the mutant cells deplete their free iron reserves more rapidly and stop growing after 1–2 duplications, whereas the parental wild type strain is able to sustain about 5–6 duplication rounds. In the case of iron overload, growth is strongly reduced in all strains, but the double mutant is more resistant to excess iron than the wild type strain and is able to grow, albeit slowly. Indeed, under the experimental conditions of this study most iron is very likely taken up via macropinocytosis, a process that is not affected by disruption of \textit{nramp1} or \textit{nramp2}. However, Nramp1 loss is expected to strongly reduce iron release from the macropinosomes to the cytosol, as iron transport across the phagosomal membrane was found to be inhibited in \textit{nramp1}-KO mutant (Peracino et al., 2006). In contrast to many mammalian cells, in \textit{Dictyostelium} the endolysosomes are not the dead-end of endosomal traffic. Instead they mature into neutral post-lysosomal vesicles that fuse with the plasma membrane, releasing their contents. Thus, if Nramp1 is inactive, iron will remain in large part entrapped in macropinosomes and could be excreted. This can explain the phenotype of both the single Nramp1 and the double Nramp1/ Nramp2 KO mutant. The higher resistance of the latter to iron overload, compared to the parental AX2 strain and to the single mutants, could depend both on less iron leaking from macropinosomes and from the contractile vacuole, due to absence of Nramp1 and Nramp2. However, disruption of both genes may also affect expression of other potential regulators of iron homeostasis, such as ferritin-like proteins, which appear to be encoded in the \textit{Dictyostelium} genome. Cloning and analysis of these genes will be required for a better understanding of iron homeostasis in \textit{Dictyostelium}.

In summary, the described phenotypes can be explained by the model presented in Fig. 8, according to which Nramp1 and Nramp2, due to their conserved function, distinct intracellular location and to colocalization with the vacuolar ATPase, synergistically regulate proton-driven iron transport across the phagosomal/macropinosomal membrane and the membrane of the contractile vacuole.

**Material and Methods**

**Cell and bacterial strains and culture methods**

\textit{Dictyostelium discoideum} parental strain AX2 was used. The Nramp1-null mutant HS860 and AX2 cells expressing Nrampl-GFP had been generated previously (Peracino et al., 2006). All strains were cultured axenically in either AX2 medium (Watts and Ashworth, 1970) or minimal medium (Franke and Kessin, 1977), with or without FeCl3. In both cases, cells were grown in Erlenmayer flasks at 23°C under shaking at 150 r.p.m. on a gyratory shaker in a climatic cabinet (Kuehner, Basel, Switzerland) as described (Bussolino et al., 1991). Blastocidin in a concentration of 10 µg/ml was added to knockout mutants. Cells expressing GFP-fused proteins were cultured in the presence of 10 or 20 µg/ml G418.

For growth and development on nutrient agar, cells were mixed with \textit{Escherichia coli} B/2, plated on nutrient agar plates containing peptone and glucose in Soerensen phosphate buffer and incubated at 23°C (Sillo et al., 2008). Growth and development were observed in a Wild MSZ stereomicroscope and pictures were taken with a Bresser PC-Microcamera 3.0 MP webcam connected to a PC-computer, using Webcam Companion 1 software.

**Legionella pneumophila** Corby expressing GFP was grown on buffered charcoal yeast extract agar (BYCE) containing 5 µg/ml chloramphenicol and incubated for 72 hours at 37°C and 5% CO2 (Fajardo et al., 2004).

**Cell development on non-nutrient agar plates**

For synchronizing development, exponentially growing cells were harvested from culture medium, washed twice in 0.017 M Soerensen Na/K phosphate buffer, pH 6.0 and resuspended in the same buffer at a concentration of 1×107 per ml. Aliquots of 0.05 ml were plated on agar plates buffered with Soerensen phosphate buffer at a density of about 3×105 cells per cm2, and incubated at 23°C (Bozzaro et al., 1987).

**Infection assays with \textit{L. pneumophila}**

Exponentially growing AX2 and mutant cells were washed, incubated in AX2 medium without maltose and aliquots of 1×105 cells were plated in 96-well tissue culture plates. Freshly collected \textit{L. pneumophila} were added at a MOI of 1:1 and immediately centrifuged at 600 g for 10 min at room temperature to synchronize infection. The number of viable bacteria was calculated by counting the colony forming units (CFU) per ml immediately after centrifugation and during the subsequent 96 hours (Bozzaro et al., 2012).

**Gene cloning, vector construction for GFP expression and cell transformation**

The \textit{nramp2} gene (DBD_0275815) was cloned starting from the genomic DNA fragment B/21 KO12 amplified with the appropriate primers and cloned in pGEM- TEasy (Promega, Madison, WI, USA), according to Sambrook et al. (Sambrook et al., 1989). For fusion with GFP, the Nramp2-coding sequence was amplified from pGEM-TEasy-Nramp2 and inserted into the EcoRI site of the pDEX-GFP vector with Nramp2(C) connected to S657-GFP by the linker EFKKKL (Westphal et al., 1997). Cell transformation was done by electroporation (Pang et al., 1999) and transformants were selected on plates with nutrient medium containing 10 µg/ ml G418.

**Construction of single \textit{nramp2} and double \textit{nramp1/2} knockout mutants**

The blastocidin cassette was excised from the pLPLBp(foxed Bsr) vector (Faix et al., 2004) in Smel, purified and inserted in the single EcoRI site of the Nramp gene in pGEM-TEasy-Nramp2. The vector carrying the selectable marker was linearized from the pGEM-TEasy vector with EcoRI, purified and electrotransported in AX2 cells. The transformed cell population was splitted in 96-well plates and grown in the presence of 10 µg/ml of blastocidin. Resistant clones were screened by PCR using appropriate primers. Positive clones were subject to Southern blot analysis as described (Bracco et al., 1997). After a preliminary phenotypic analysis of three clones, one clone was treated with pDEX-NLS-cre to transiently express the CRE recombinase and remove the blastocidin cassette (Kimmel and Faix, 2006). To generate the double mutant, one clone was further transformed with the linearized Nramp1 gene containing the bsr cassette from the Nramp1-KO vector (Peracino et al., 2006). Clones were analyzed by PCR and positive ones further confirmed by Southern blot analysis.

**Gene expression analysis by quantitative PCR**

Total RNA was extracted with TRIzol, according to manufacturer instructions (Invitrogen, Carlsbad, CA, USA) from cells at different developmental times and analyzed by quantitative PCR The RNA was treated with Dnase and retrotranscribed with random hexamer primers and Multiscribe Reverse transcriptase (High Capacity cDNA Archive Kit; Applied Biosystems, Foster City, CA, in accordance with manufacturer’s instructions. Expression levels of \textit{nramp1}, \textit{nramp2} and reference gene \textit{histone H3} were evaluated with SYBR technology with optimized PCR conditions and primer concentrations. Primer sequences were as follows: \textit{nramp1} FW: CTCACGGGTACGCCAAGTGATT, \textit{nramp1} RW: ATTACCAATGGGCGCGCGAAG, \textit{nramp2} FW: CGAACGCAGTCTCAGCGAA, \textit{nramp2} RW: GCAGCTATCATCACATCATCGTC, \textit{H3} FW: GGCGCCTGCTAGCTGCA, \textit{H3} RW: GGGACAGCAATGCTGTCG.

Melt curve analysis and efficiency evaluation were performed for all the amplicons. Quantitative PCR (qPCR) was carried-out on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) in 384-well plates assembled by Biorobot 8000 (Qiagen, Germantown, MD, USA). Baselines and thresholds for Ct calculation was set up manually with the ABI Prism SDS 2.1 software.

**In vivo microscopy, fluorescence imaging and immunofluorescence**

Living cells expressing \textit{Nrampl(C)-} or \textit{Nrampl(2C)-GFP} were incubated with 1 mg/ml TRITC-dextran for 5 min under shaking, washed and plated on 6×6 cm glass coverslip equipped with a 5-cm diameter Plexiglas ring. For staining acidic vesicles, cells were incubated with 0.1 µM Neutral Red. Confocal series images were taken on an inverted Zeiss LSM510 microscope as described (Peracino et al., 2006). For immunofluorescence studies, cells were fixed with cold methanol, incubated with antibodies against the V-H+-ATPase subunit Va and mounted with Gelvatol as described (Peracino et al., 1998). TRITC-labeled goat anti-mouse...


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