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METALS AND MEMBRANE METAL TRANSPORTERS IN BIOLOGICAL SYSTEMS: THE ROLE(S) OF Nramp IN HOST-PARASITE INTERACTIONS

Zhuoer Lin[§], José-Antonio Fernández-Robledo[§], Mathieu F. M. Cellier[†] and Gerardo R. Vasta[§]*

[§]*Center of Marine Biotechnology, University of Maryland Biotechnology Institute, 701 East Pratt Street, Suite 236, Baltimore, Maryland 21202-3101, U.S.A.*

[†]*INRS-Institut Armand-Frappier, 531 Bd. des Prairies, Laval, PQ, Canada H7V 1B7*

***E-mail**: *vasta@umbi.umd.edu* Phone: (410) 234-8826; Fax: (410) 234-8896

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Review Article

Abstract

Metals are essential to a wide variety of biological processes in both prokaryotic and eukaryotic systems. Thus, a fine-tuned system for metal acquisition, storage, and export is critical for most biological activities, and disruption of metal homeostasis can cause dysfunction, disease, or death. For example, iron is critical for cell growth, oxygen utilization, enzymatic activity, and innate immune responses. Concerning the latter, "nutritional immunity" has been defined as the dynamic interaction between pathogens and hosts, including sequestration of iron and other cations as a non-specific host response to infection. Hence, innate resistance to microbial challenge is, at least in part, derived from basal metabolic functions, and influenced by genetic factors. One illustrative example is the natural resistance-associated macrophage protein 1 (Nramp1), a critical factor in the mouse innate resistance to infection by intracellular pathogens, such as *Mycobacterium bovis*, *Leishmania donovani*, and *Salmonella typhimurium*. In this review, following a brief introduction about the general roles of metals and metal transporters in biological systems, we place particular emphasis on the molecular, structural, phylogenetic, and functional aspects of Nramp as a divalent cation transporter in host-parasite interactions.

Keywords: metals, biological activity, toxicity, homeostasis.

Resumen

Los metales son esenciales para una amplia variedad de procesos biológicos en sistemas procariotas y eucariotas. De esta manera un buen sistema para la adquisición, almacenaje, y exportación del metal es crítica para la mayoría de las actividades biológicas, y la interrupción de la homeostasis del metal puede causar la disfunción, la enfermedad, o la muerte. Por ejemplo, el hierro es crítico para el crecimiento de la célula, la utilización del oxígeno, la actividad enzimática, y las inmunorrespuestas innatas. En referencia a esto último, la "inmunidad alimenticia" se ha definido como la interacción dinámica entre los patógenos y los huéspedes, incluyendo el secuestro del hierro y de otros cationes como respuesta no específica del huésped a la infección. Por lo tanto, la resistencia natural al ataque microbiano se encuentra, por lo menos en parte, derivada de funciones metabólicas básicas, e influenciada por factores genéticos. Un ejemplo ilustrativo es la natural resistencia asociada a la proteína 1 (Nramp1) del macrófago, un factor crítico en la resistencia natural del ratón a la infección por patógenos intracelulares, tales como el *Mycobacterium bovis*, *Leishmania donovani*, y *Salmonella typhimurium*. En esta revisión, después de una breve introducción sobre el papel general de metales y de transportadores del metal en sistemas biológicos, ponemos particular énfasis en los aspectos moleculares, estructurales, filogenéticos, y funcionales de la Nramp como transportadora de cationes bivalentes en interacciones anfitrión-parásito.

Palabras clave: metales, actividad biológica, toxicidad, homeostásis.

Introduction

1. Metals and metal transporters in biological systems

1.1 *Biological properties of metals*

Metals are critical factors on various aspects of biological activity in both prokaryotic and eukaryotic organisms. Some metals including iron, manganese, copper, zinc, nickel, and cobalt are essential at the appropriate concentrations, but may become toxic beyond these levels. Sodium, potassium, and calcium are abundantly available in the environment and can be found at relatively high concentrations in biological systems. In some environments, however, the reduced availability of metals that function as trace elements, such as zinc, iron, manganese, cobalt, and copper, has led to the selection of efficient uptake mechanisms by organisms that must compete for them in their particular niche. Nevertheless, both categories of metals are essential to the basic environment for multiple biochemical processes and homeostatic balance. On the other hand, arsenic, lead, mercury, and silver are not required for metabolic activity, and may be toxic to the cell even at very low concentrations.

In spite of contrasting levels of sodium (10,781 ppt) and potassium (402 ppt) in the marine [1] and fresh water (<0.5 ppt) environments, the higher organisms that inhabit both media maintain their internal free sodium ion concentrations constant both intracellularly and in the circulating body fluids. Similarly, terrestrial organisms keep relatively stable sodium and potassium levels, and in humans, even the smallest deviations are recognized as symptoms of dysfunction or disease. Sodium and potassium are central to osmotic control and electrolytic balance, integrity of macromolecules and cell structures, uptake of organic metabolites, and overall cell homeostasis [2]. Like sodium and potassium, magnesium is also abundant in seawater (1,284 ppt) but is present at relatively low levels in fresh water. Thus, magnesium must be rejected or excreted by marine organisms, but internalized by fresh water organisms to maintain the free magnesium concentration at around 10-3 M in most cells and body fluids. The cross-linking of carboxylated and phosphorylated anionic polymers via magnesium is essential to the integrity of the cell outer surfaces, a structural role shared with calcium ions, which act cooperatively to stabilize external cell structures. Anionic centers are also neutralized by magnesium, thereby enabling their binding, protecting them from hydrolysis, and facilitating their chemical reactions. Magnesium plays a role

in stabilizing and energizing various types of biomolecules including nucleic acids, lipids, proteins, and polysaccharides. Further, given the central role of phosphate in polymer assembly, metabolism (DNA, RNA, protein, polysaccharide, and lipid synthesis), intracellular signaling, and bioenergetics, it becomes evident that cells have evolved with magnesium as the essential anion partner, with a dramatically increased value during the emergence of multicellular organisms [3]. Similarly, the role of calcium in advanced biological systems has been well characterized. Among its multiple functions, calcium is essential to the control of metabolic pathways, mechanical stability of cell walls and membranes, contractility of filaments, fertilization, cell division, and hormonal activities [4].

Trace metals function as co-factors in enzymatic reactions, but can also play an important role in enzyme structure stabilization without involving enzymatic activity. Zinc is relatively abundant in seawater compared to copper, iron, cobalt, or cadmium, and it is one of the most available and common of the trace elements. Zinc is a multi-functional element found in almost 300 enzymes, and is involved in catalytic, co-catalytic, and/or structural functions; enzymes containing zinc in the reactive center are widespread in nature [5, 6]. Zinc finger motifs, which are devoid of enzymatic activity, are only found in eukaryotes. Some of the zinc fingers bind to DNA promoter sites, whereas others appear to have a structural role in critical enzymes [7]. In contrast, free iron is often involved in redox chemistry and reactions often involve production of free radicals, either as electron-transfer intermediates, the enzyme's ground state, or as part of the substrates' reactions [8-10]. Although considered as highly relevant in biological systems, the role(s) of manganese remain poorly explored [11]. Its involvement in O2 release by photosynthesis system II, however, has recently generated substantial interest, as well as the manganese co-factored superoxide dismutases (SOD) of both prokaryotes and eukaryotes [4]. The relevance of iron and manganese as critical factors in infections by intracellular pathogens will be discussed in the second part of this review.

Despite the beneficial roles played by metals, some transition metals (*e.g.* iron) can be toxic at high concentration by triggering oxidative stress. Heavy metals, however, can be toxic even at low levels, by becoming alternative substrates for membrane transporters. The toxicity of lead is due to its ability to mimic other biologically important metals, most notably calcium, iron, and zinc, which act as cofactors in many enzymatic reactions [12]. Although lead binds to and interacts with many enzymes, it does not properly function as a cofactor, thus interfering with the enzyme's ability to catalyze its normal reaction(s). In plants, lead accumulates in the photosynthesis system (PS) II, damages its secondary structure, and reduces energy transport to chlorophyll a [13]. In humans, lead poisoning may cause irreversible neurological damage, and have serious detrimental effects on renal, cardiovascular, and reproductive function.

1.2. *Breakdown of metal homeostasis*

Because metals play such significant roles in a vast array of biological activities, fine-tuned acquisition, storage, and release systems are critical to the normal function of any single organism. As a major component in the mineralization of shells and bones, calcium is the most abundant metal by mass in many invertebrates and most vertebrates. Due to its critical role as a second messenger in many cell types [14], calcium oscillations can effectively trigger apoptosis under certain conditions, and disruption of calcium signaling may cause cell death [15]. Perturbed neuronal calcium homeostasis is implicated in age-related cognitive impairment and Alzheimer's disease. Aging neurons encounter increased oxidative stress and impaired energy metabolism compromising the function of proteins related to membrane excitability and subcellular calcium dynamics. Toxic forms of amyloid beta-peptide (Abeta), which is the main constituent of amyloid plaques in the brain of Alzheimer's disease patients, can induce calcium influx into neurons by inducing membrane-associated oxidative stress or by forming oligomeric pores, thereby rendering neurons vulnerable to excitotoxicity and apoptosis [16]. This effect can be exacerbated by increased iron

levels, which contribute to oxidative stress and mitochondrial damage, leading to neuronal death [17]. Thus, metal chelation [18] and uptake inhibition via membrane transporters [18] represent potentially valuable therapeutic approaches to such disease states.

The vertebrate evolutionary process has selected iron as the oxygen carrier, which together with other transition metals such as copper and manganese are vital components of redox reactions because they can readily gain or lose electrons. Although iron is a relatively abundant element in nature, it is estimated that more than 2 billion people worldwide suffer from iron deficiency anemia [19-21]. Iron deficiency results in impaired production of iron-containing proteins, the most prominent of which is hemoglobin. Cellular iron deficiency inhibits cell growth, and subsequently leads to cell death. Conversely, hemochromatosis is an inherited disorder that results in dysregulated absorption of iron, which builds up in tissues eventually leading to organ damage derived from free radicals toxicity [10]. Further, excess iron increases cancer risk, presumably due to oncogene mutation via generation of reactive oxygen species [22].

1.3. *Membrane metal transport proteins*

A variety of cellular mechanisms have evolved towards the control of metal toxicity, including enzymes that neutralize radicals, intracellular metal chelators (*e.g.* ferritin and transferrin) or chaperones (*e.g.,* mitochondrial frataxin Fe chaperone [23], and cytosolic Fe chaperone [24]), and a strict control of transmembrane metal transport. Membrane transporters are critical components of the homeostatic systems necessary for maintaining a balance between the amounts of metal required for biological processes and those that might be toxic. Among these, detoxification systems export any transition metals that may be present at excessive levels. In yeast, several metal detoxification systems are operative: the PMR1, an P-type ATPase pump, transports manganese into the Golgi, and is ultimately exported from the cell via secretory pathway vesicles. In addition, manganese detoxification is achieved through a vacuolar ATPase that traps the metal in the lumen of the vacuole [25-28]. Further, the cation diffusion facilitators (CDF) constitute a family of proton antiporters that can accumulate Zn in the vacuoles in an ATP-dependent manner [29]. It is noteworthy that in *E. coli*, a CDF homolog is involved in iron-efflux. Most metal transport systems have narrow specificities, and only transport one or very few metals. However, cells possess multiple, genetically separable transport systems, sometimes with overlapping specificity that ensures the required uptake of metals even under adverse physical conditions [30].

Membrane transport proteins, including those that transport metals, are currently classified by the Transport Classification (TC) system (http://www.tcdb.org/) [31, 32], and approved by the transporter nomenclature committee of the International Union of Biochemistry and Molecular Biology. In the TC system each functionally dissimilar transporter is identified by a five-digit TC number: the first of these digits (a number) refers to the class, as revealed by the mode of transport and energy coupling mechanism; the second (a letter) refers to the subclass, determined by the type of transporter and the energy coupling mechanism; the third (a number) refers to the family or superfamily; the fourth (a number) refers to a phylogenetic cluster within the family (or a family within the superfamily); and the fifth (a number) refers to the substrate specificities of the individual transporter. There are four mayor types of transporters in biological systems based on the type and direction of transport and the energy coupling mechanism [32]: Channel-type transporters, carrier-type transporters, primary active transporters, and phosphoenolpyruvate -dependent and phosphoryl transfer-driven group translocators (Fig. 1). Channel-type transporters fall into subgroups such as α-Type channels, β-Type channels, pore-forming toxins, non-ribosomally synthesized channels, holins, paracellular channels, viral fusion pores (http://www.tcdb.org/), and aquaporins [33]. The Slc (Solute carrier) series belongs to the electrochemical potential-driven transporters, and include 43 families and 298 transporter genes. Members of Slc series can function as exchanger, passive transporter, and coupled-transporter, and they may be present in the plasma,

vesicular, or mitochondrial membranes [34]. As recently reviewed, different types of transporters have more or less restricted taxonomic distribution. In bacterial membrane-transport systems, single gene-encoded proteins are mainly energized via ATP, whereas secondary transport proteins usually use H+ as a co-transport molecule. In contrast, animals are particularly divergent in their channel protein genes, and plants have larger numbers of P-type ATPase and secondary active transporters. Furthermore, in both plants and animals the secondary transporter genes have diverged with regards to their co-transporter molecules. Animals use Na+ for the generation of concentration gradients across plasma membranes, that are dependent on secondary active transporters and membrane voltages that in turn are dependent on ion transport regulation systems. Plants use proton pools in vacuoles and the apoplast, and similarly to animals, the proton gradients are dependent on secondary active transporters [35].

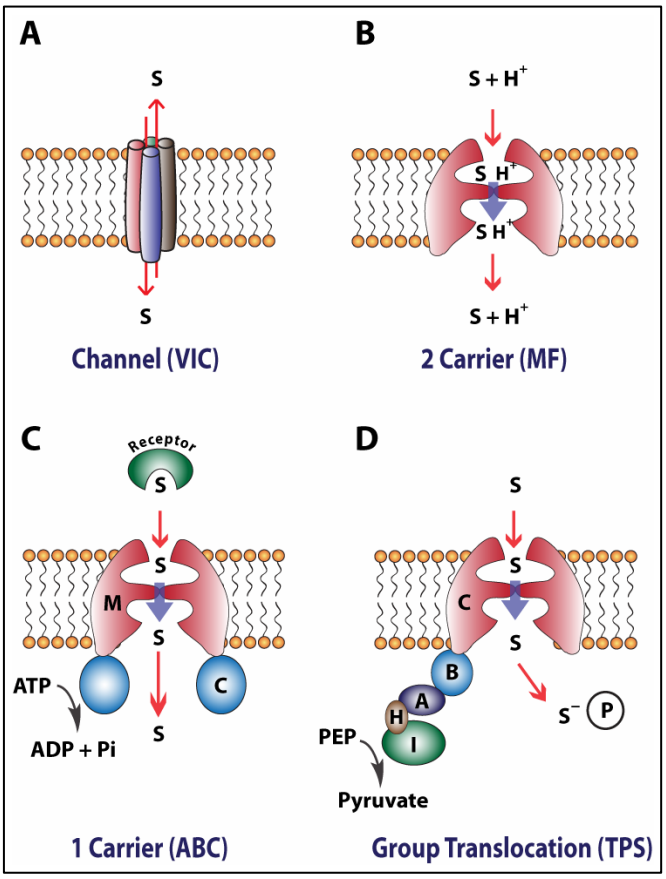

Figure 1. Schematic of the four major types of transporters in biological systems. (Based on [32]). A. Channel-type transporters. These transporters enable facilitated diffusion through a transmembrane pore or channel with no evidence of the intervention of any carrier-mediated mechanisms. B. Carrier-type transporters. This transporter type requires a carrier-mediated process to transport the substrate and they are coupled to chemiosmotic energy. In uniports, a single species is transported; in antiporters, two or more species are transported in opposite directions, in symporters, two or more species are transported in the same direction. C. Primary active transporters. Usually multidomain, these transporters are driven by pyrophosphate hydrolysis. Depicted is an ATP-binding cassette (ABC) carrier; the solute (S) is fed into the membrane channel (M) by a receptor. D. PEP-dependent, phosphoryl transfer-driven group translocators. These multidomain transporters modify the transported species during the transport; delpicted group translocators (PTS) where the sugar substrate is phosphorylated during transport.

1.4. *Dysfunction of membrane metal transport proteins and disease*

Numerous metal transporters have been characterized based on their substrate specificity, transport efficiency, and other functional properties; their dysfunction is usually associated to disease. The great effort put in research of the sodium pump derives from its role in catalyzing adenosine 5'-triphosphate hydrolysis by forming a phosphorylated enzyme intermediate and coupling the energy released to unequal counter transport of sodium and potassium ions. Besides the fundamental roles in energy utilization, the ion gradient generated by the pump is important for a variety of secondary physiological processes ranging from metabolite transport to electrical excitation of nerve and muscle [36]. Sodium ion transporters in the sarcolemma are involved in numerous vital cell functions, including excitation-contraction coupling, energy metabolism, pH and volume regulation, development, and growth. In a number of cardiac pathologies, the intracellular sodium concentration is elevated [37]. Voltage-gated sodium channels transmit electrical signals by action potential in excitable cells (*e.g.* neurons) and propagate the signal in the peripheral and central nervous systems. Therefore, it is not surprising that more and more evidence indicated that mutations, changes in expression, or inappropriate modulation of these channels can lead to electrical instability of the cell membrane and inappropriate spontaneous activity observed during pathological states [38].

Calcium dysregulation caused by a novel calcium-conducting channel named CALHM1 (calcium homeostasis modulator 1) has been identified as a genetic link to the disorder in Abeta production, and the pathogenesis of Alzheimer's disease. Calcium homeostasis caused by CALHM1 has also been shown to be perturbed in dendritic spines adjacent to amyloid plaques [39].

The cardiac isoform of the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA2a) is a calcium ion pump powered by ATP hydrolysis. SERCA2a transfers calcium from the cytosol of the cardiomyocyte to the lumen of the sarcoplasmic reticulum during muscle relaxation. Decreased SERCA2a expression was detected in human heart failure, and considering the key role of SERCA2a in cardiomyocyte calcium regulation, this can lead to abnormal calcium dynamics, a deficient contractile state, and finally heart failure [40].

Pulmonary arterial hypertension (PAH) patients demonstrate many cellular abnormalities linked to potassium channels. This pathological linkage is due to the essential role of potassium channels in regulating the pulmonary artery smooth muscle cell (PASMC) population in the pulmonary vasculature, in cell apoptosis, survival and proliferation. In PAH, PASMCs exhibit downregulated expression of various potassium channels and apoptosis inhibition [41].

2. Nramp as a divalent cation transporter in metal homeostasis and host-pathogen interactions

The concept of "nutritional immunity" defines the dynamic interaction between pathogens and hosts, including the competition for essential nutrients such as small organic molecules, amino acid, fatty acids, nucleotides and other co-factors [42]. Hence, the innate resistance to infection by intracellular pathogens is, at least in part, derived from basal metabolic functions, and influenced by genetic factors [43]. An instructive example for nutritive host–pathogen competition is represented by the mutual requirement for iron, manganese and potentially other divalent cations. One illustrative example is the divalent cation transporter Nramp (Natural resistance-associated macrophage protein), which plays roles in both trace metal acquisition and natural defense against intracellular pathogens.

2.1. *Identification of Nramp as a marker for resistance to infection*

Nramp was identified from a survey of inbred mouse strains in a model of *Salmonella typhimurium* infection. Resistant (LD100>104-105 bacteria) or susceptible (LD100<100 bacteria)

phenotypes to *Salmonella* infection were controlled by a single locus (*Ity*) [44]. Similarly, another group showed independently that the replication of *Leishmania donovani* in mouse tissues was either allelic or tightly linked to *Lsh* [45]. A then third locus (*Bcg*) was also mapped to the same region of mouse chromosome 1 (*Bcg/Lsh/Ity* locus)[46], in *in vivo* studies of replication of mycobacteria [45, 47-49]. In each case the resistant allele that permitted restriction of intracellular replication of the infectious agents was shown to be dominant. By positional cloning the chromosomal region covering the *Bcg/Lsh/Ity* locus was found to encode six candidiate genes, and was expressed exclusively in spleen, liver, and macrophages extracted from them[48].. The highly hydrophobic integral trans-membrane protein encoded by this mRNA was reminiscent of a transporter or an ion channel. Later studies rigorously demonstrated that *Nramp1* (*Bcg/Lsh/Ity* gene or Slc11, Solute carrier 11) was involved in host resistance to intracellular infections. Interestingly, it was a single Gly169Asp substitution in predicted transmembrane segment (TMS) 4 of the protein that was responsible for the susceptibility trait. These findings spiked the search for Nramp in humans where it was also shown to be associated with resistance to infection with a variety of intracellular pathogens, becoming a promising marker for resistance to intracellular pathogens. Prompted by this possibility, strong interest was generated in searching for this marker in agriculturally important animal species such as of chickens and cattle, to increase the overall level of genetic resistance by using selective breeding programs [50-52]. Pursuing the same objectives, the *Nramp* gene has also been described in numerous wild and farmed fishes [53-58].

From this point on, in the section titles, subtitles and general statements made in the remainder of this review, all Nramp family members, including Nramp isotypes, Slc11, DMT1, SMF1/2, MntH, and others, will be referred to (*sensu lato*) as Nramp.

2.2. *Nramp functions in metal homeostasis*

Functional studies revealed that Nramp homologs are proton-dependent divalent metal transporter with a high affinity for Mn2+ and Fe2+. Heterologous expression studies of Nramp family members from various organisms have identified several metal substrates (Table 1) by either electrophysiology studies in *Xenopus* oocytes or yeast complementation. Besides the mammalian Nramp1, the Nramp isotype, Nramp2, or divalent metal transporter 1 (DMT1) was identified and characterized [59]. A mutation (G185R) at the Nramp2 locus causes microcytic anemia and iron deficiency in the *mk* mouse and the Belgrade *(b)* rat [60, 61], a pathology associated with decreased iron uptake in the duodenum, and impaired iron metabolism in peripheral tissues. Nramp2 is believed to function as the major transferrin-independent iron uptake system at the intestinal brush border, and in the transport of transferrin iron across the membrane of acidified endosomes as part of transferrin cycle [62].

Nramp homologs are ubiquitously present in virtually all taxa. In yeast, the Nramp homologs SMF1 and SMF2 transport Mn2+, but also Cd2+, Cu2+ and Co2+ at lower rate, while SMF3 is presumed to mostly transport Fe2+ [63]. The fruit fly *Nramp* homologue, *malvolio*, is expressed primarily in the brain, and mutations at this locus cause a sensory-neuron defect in taste discrimination. The mutant phenotype can be corrected by dietary Fe2+ or Mn2+, and by expression of mammalian *Nramp1* in *malvolio* transgenic flies [64]. The plant Nramp family has been well documented in both genomic and EST (expressed sequence tags) databases, demonstrating that genes from this family are present in virtually all plants studied at the molecular level. Functionally, plant Nramp genes complement yeast mutants deficient in the uptake of several metals, including iron, manganese and zinc, suggesting that their function as metal transporters is conserved across various taxa. *Schistosoma mansoni*, an intravascular human parasite with a high nutritional and metabolic demand for iron, has two Nramp homologues (SmDMT1A and SmDMT1B) with different expression patterns and subcellular compartmentalization. SmDMT1

localizes primarily to the tegument, suggesting that the parasite uses this transporter for iron acquisition [65].

Table 1. Selected properties of Nramp homologs (expanded from [94]).

Organism	Protein	% identity to mouse Nramp1	Substrate(s)*	Mutant phenotype
Mouse	Nramp1	100	**Mn^{2+}**, Fe^{2+}	Susceptibility to infection
	Nramp2	78	**Fe^{2+}**,Mn^{2+},Zn^{2+}, Co^{2+},Cd^{2+}, Ni, Pb	Microcytic anemia
Human	Nramp1	79	ND	Susceptibility to infection/ autoimmune disease
	Nramp2	79	**Fe^{2+}**, Mn^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+}, Cd^{2+}, Ni, Pb	Unknown
Dog	Nramp1	87	ND	Susceptibility to *Leishmania*
Saccharomyces cerevisiae	Smf1	42	**Mn^{2+}**, Co^{2+}, Cu^{2+}, Cd^{2+}	No growth on EGTA/Alkaline pH
	Smf2	43	**Mn^{2+}**, Co^{2+}, Cu^{2+}, Cd2+	No growth on EGTA/Alkaline pH
	Smf3	45	**Fe^{2+}**, $Mn2^{+}$	ND
Escherichia coli	MntH	37	**Mn^{2+}**, Fe^{2+}, Zn^{2+}, Cd^{2+}, Ni^{2+}	No growth on metal chelators
Mycobacterium tuberculosis	Mramp	30	Mn^{2+}, Fe^{2+}, Zn^{2+}, Cu^{2+}	ND
Zebrafish	Cdy/Nramp2	73	**Fe^{2+}**	Chardonnay (iron deficiency)
Drosophila melanogaster	Malvolio	70	Fe^{2+}, Mn^{2+}	Malvolio, behavioral taste defect
Arabidopsis thaliana	At-Nramp1,2,3,4,5,6	28	Fe^{2+}, Cd^{2+}, Mn^{2+}	ND
	EIN2-Nramp	21	Unknown	Triple response to ethylene
Mizuhopecten yessoensis	scDMT	55	**Ca^{2+}**, Fe^{2+}, Cd^{2+}	Shell formation
Schistosoma mansoni	SmDMT1A, -B	50	Fe^{2+}	ND

*****In bold: preferred substrate for transport; ND: no data.

2.3. *Nramp as a potential microbial virulence factor*

Because iron and other divalent cations are necessary for the growth of microbial pathogens and parasites, their metal transporters have been investigated as candidate virulence factors. *Perkinsus* species are parasites of oysters, abalones, clams, and scallops, and have caused substantial damage to these fisheries worldwide [66, 67]. *Perkinsus marinus* is a facultative intracellular parasite that causes "Dermo" disease in the eastern oyster *Crassostrea virginica* [68]. In the past few decades it has produced extensive damage to oyster bars along the Gulf of Mexico and Atlantic coast of North America, with catastrophic consequences for local fisheries and the health of coastal waters [69]. Intensity of *P. marinus* infection in *C. virginica* increases with environmental iron concentrations in a dose-dependent manner (Fig. 2). Like most intracellular pathogens, once inside the host *P. marinus* must acquire trace elements such as iron, which are essential for growth, DNA synthesis, electron transport, energy metabolism, and enzyme activity [70]. Indeed, chelation of environmental iron with desferrioxamine (DFO) significantly inhibits *P. marinus* growth [71] and a putative divalent cation membrane transporter identified as an Nramp homolog, may be responsible for iron uptake by this parasite [72].

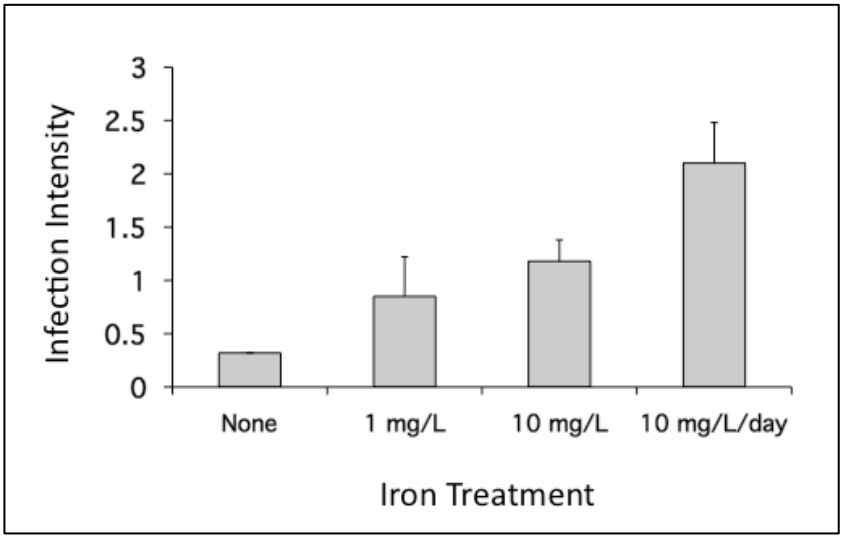

Figure 2. Effect of environmental iron on *Perkinsus* infection intensity. Oysters were held in aquaria and subjected to different iron (Cl2Fe) regimes and the *Perkinsus* prevalence evaluated by incubating oyster hemocytes in fluid thyoglycolate medium (FTM) [93]. Iron overload (10 mg/L added daily to the aquaria) resulted in increase of Dermo infection intensity (J.D. Gauthier and G. R. Vasta, unpublished).

The bacterial Nramp homologs MntH (H+-dependent Mn transporter) are widespread. Disruption of *MntH* in *Escherichia coli* and *S. typhimurium* does not affect bacterial growth under aerobic conditions in minimal or rich medium, implying that MntH is not essential for growth under normal laboratory conditions [73, 74], and at least one potentially redundant manganese acquisition system (*e.g.* ATP-binding cassette transporter) is present in pathogenic enterobacterial microorganisms [75]. The lack of a strong growth phenotype suggests either that Mn2+ is not critical for growth or that other enterobacterial transporters can compensate for the loss of MntH-mediated uptake. However, elimination of the Gram positive *B. subtilis mntH* gene prevented bacterial growth in Mn-limited medium, implying that bacterial physiology influences *mntH* phenotype [75]. It was demonstrated in *E. coli* that overexpression of *mntH* from a plasmid could restore growth of the temperature sensitive *hflB1* mutant, which requires high intracellular metal ion

concentrations to grow at non-permissive temperatures [75]. In addition, when MntH was overexpressed, *E. coli* cells become more sensitive to Mn2+, Cd2+, Co2+, Fe2+, Ni2+, and Cu2+ and direct measurements of radio-label uptake showed that *E. coli* MntH has higher affinity for Mn2+ [74]. Likewise, it was confirmed that overexpression of *mntH* renders both *E. coli* and *S. typhimurium* more sensitive to growth inhibition by Mn2+ and Cd2+, and that the loss of *mntH* rendered them more sensitive to hydrogen peroxide but not to superoxide. Although bacterial pathogens acquire host iron and other divalent metals via multiple routes, Nramp homologs (MntH) identified in *M. tuberculosis* [76], *M. leprae* [77], and *Salmonella* spp. [73] represent potential virulence factors.

2.4. *Three-D structure and phylogenetic analysis of the Nramp family*

Most Nramp family members are transmembrane proteins consisting of 10 to 12 TMS encompassing > 400 amino acids. [78]. No successful attempts to crystallize Nramp have been reported to date and a three-dimensional model structure of this transporter has been obtained by a combination of homologous modeling and selected mutation assays [79]. Genetic studies using prokaryotic and eukaryotic Slc11 homologs and various topological reporters have yielded a consensus transmembrane topology that places both ends of the Slc11 hydrophobic core (TMS 1-10) on the cytoplasmic side of the membrane [80-82]. Homology structural modeling combined with biochemical studies on the *E. coli* MntH further supported this global topology, and suggested that Slc11 carriers may share a structural fold that has been solved for apparently unrelated families of Na+-dependent transporters showing less than 15% overall amino acid sequence identity (Slc 6, [83], Slc 5, [84], and Slc23, [79, 85]). Thus, the tridimensional model for the Slc11 hydrophobic core comprises two domains that are direct repeats with inverted transmembrane topology. The two Slc11-specific triplets Asp-Pro-Gly (TMS1) (highlighted in red in Fig. 3) and Met-Pro-His (TMS6) (highlighted in magenta in Fig. 3) would occupy the central position and together with segments of TMS3 and TMS8, form a three-dimensional arrangement enabling directional cation symport [83-85]. The TMS1 DPG motif contributes to proton-binding and -motive force, shown by the loss of H+ uptake in *E. coli* MntH Asp34 mutants, while the TMS6 MPH motif mediates pH-dependent regulation consistent with requirement for *E. coli* MntH His211 for Cd2+ uptake at neutral pH. These two Slc11-invariant sites were accessible *in situ*, respectively to fluorescein-maleimide and N-ethyl maleimide [79].

Although Nramp was first indentified in the mouse, members of this protein family are present in most eukaryotes and bacteria investigated so far. Phylogenetic analysis of the Nramp family suggests that several steps may have taken place along its evolution. Nramp family mostly likely evolved from bacterial sodium- or proton-motive substrate symporters to metal nutrient-specific transporters. The most ancient subgroup of Nramp is MntH B, which gave rise to MntH A that evolved into the eukaryotic prototype Nramp, which by gene duplication resulted in the archetype Nramp. In addition, horizontal gene transfer led to the unique subgroup MntH C (Cα, Cβ, Cγ), with both eukaryotic and prokaryotic Nramp features. Eukaryotic Nramp are taxonomically diverse, with potential homologs in bikonts (plants, and chromoalveolates) and unikonts (amebozoans, fungi, and animals) [72, 86] (Fig. 4); the Nramp archetype subgroup I is found only in monocot and eudicot plants, and the subgroup II is shared by both unikonts and bikonts, while prototype Nramp are restricted to amebozoans and fungi, green or red algae and lower plants, suggesting a selective loss of this isoform in animals, higher plants as well as in chromoalveolates. [86].

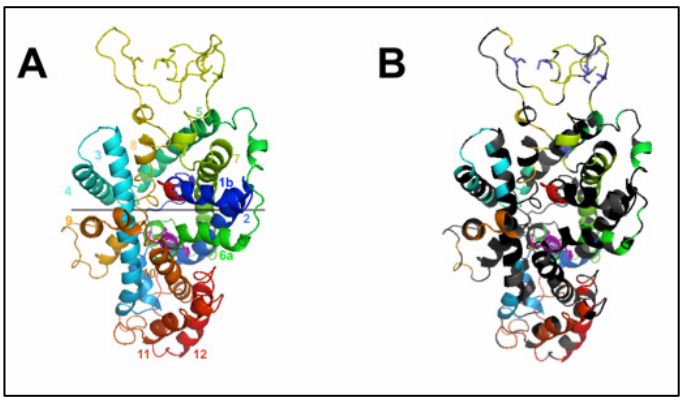

Figure 3. Homology model of the structure of PmNramp1. View from the outside the cell of the Mdh1 (Slc23) -based Slc11 model obtained by threading. The model corresponds to an open-to out- conformation. A. Numbers 1–5 and 6–10 designate the TMS forming the inverted domains and the half-helices 1a and 6b are placed at the *bottom*, orthogonal to the membrane plan [79]. TMS 1 and TMS6 appear close to each other and, together with TMS 3 and 8, lining a central water accessible cavity. The Slc11-specific sites in TMS 1 and TMS6 that contribute to cations binding are highlighted in red and magenta, respectively. B. Residues that are identical among PmNramp1-3 are indicated in black, except a cluster of conserved extracellular Cys found in the loop between TMS 7 and 8, indicated in light blue; residues conserved in PmNramp1, 2 are indicated in grey. The most conserved regions of PmNramp appear to be TMS1 and 6, 3 and 8 as well as TMS10, consistent with the known molecular data for the cation-driven symport mechanism described for the distantly related LeuT-Slc6 family.

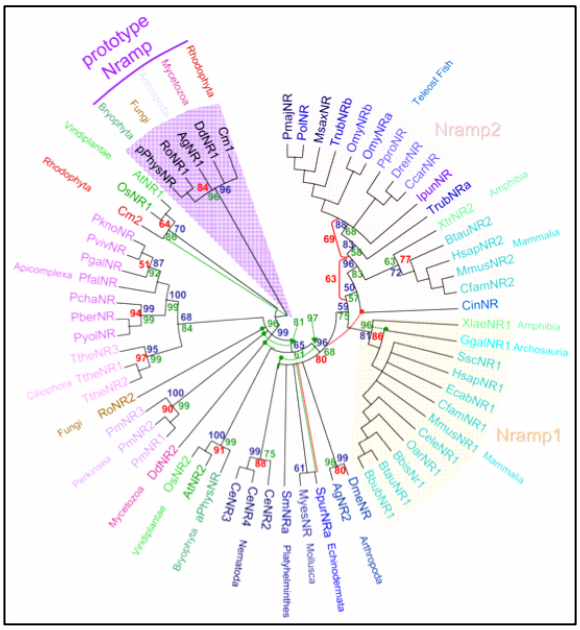

Figure 4. Dendrogram of the Nramp family. This dendrogram was obtained using 63 eukaryotic sequences of the Nramp family, including 5 "prototype" Nramp, which were aligned as previously described (61). A set of 328 parsimony-informative sites (at least 2 different amino acids (aa) each represented at least twice) was generated and analyzed by the method of Maximum Parsimony, or used to calculate with the substitution matrix Blosum 62 a continuous gamma distribution to model site-specific heterogeneity of aa replacement rate among sites, which was used for Minimum Evolution and Maximum Likelihood analyses (61). The level of statistical confidence (determined by bootstrapping, 4-6000 replicates) is indicated in percent for each node (green, Maximum Parsimony; blue, Minimum Evolution; and red, Maximum Likelihood); alternative node topologies are indicated with dotted lines.

2.5. *A "tug-of-war" for iron through host and pathogen Nramp homologs*

Two different theories have been proposed about how the host Nramp1 functions in defense against intracellular pathogens. One theory implies that Nramp1 might increase intraphagosomal Fe2+, and through the Haber-Weiss/Fenton reaction facilitates the generation of microbicidal reactive oxygen species [87, 88]. The second suggests that Nramp1 deprives the intraphagosomal pathogen of Fe2+ and other divalent cations critical for growth and for the pathogen's effective antioxidant defense [89-91]. Accumulating evidence further supports the prime role of Nramp as a first line defense that limits essential metal availability to intracellular pathogens, not only in animal hosts but also in amoeba and plants [92]. In *M. tuberculosis*, it has been suggested that the prokaryotic Nramp homolog (MntH) might be in direct competition with the host Nramp for iron and other divalent cations [76]. A proposed "tug-of-war" for iron between oyster and *P. marinus* Nramp is illustrated in Fig. 5, in which the host Nramp1 mediates efflux of divalent cations (including Fe2+ and Mn2+) from inside the phagosome and into the cytoplasm. Acidification of the phagosomal space by vacuolar H+/ATPase would provide the proton gradient as the driving force for metal efflux. Deprivation of Mn2+ and Fe2+ could deplete the parasite of nutritional metals, prevent success of individual survival strategies (virulence factors) and/or disable the pathogen-encoded detoxifying enzymes (SOD, APX, among others). The microbial archetype Nramp homologs likely function by a similar mechanism, both for the acquisition of metals from the environment, and in the competition for the same substrate(s) with their host counterpart(s)

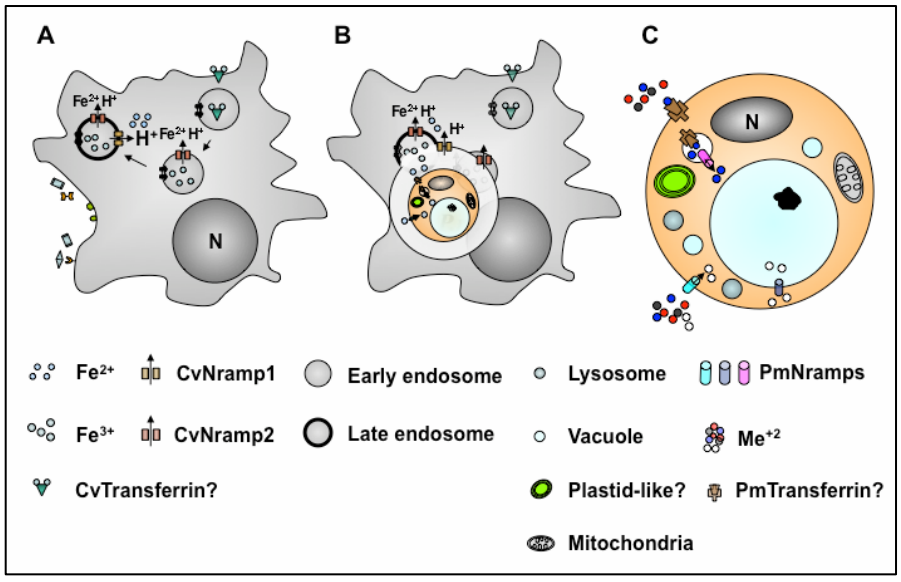

Figure 5. Model of the "tug-of-war" for iron in the *Perkinsus*/oyster system. Oyster Nramp iron uptake increases intraphagosomal Fe2+ and through the Haber-Weiss/ Fenton reaction generates bactericidal reactive oxygen species (ROS) against *Perkinsus* trophozoites. *Perkinsus* trophozoites would use Nramps to uptake iron from the parasitophorous vacuole, indirectly abrogating the production of ROS and at the same time full filling the need for iron. *Perkinsus* has three Nramp isotypes yet to be localized. Both host and parasite may also possess Nramp -independent iron uptake mechanisms (*e.g.* ferritin).

Conclusions and future directions

Metal homeostasis is critical to multiple functions in both prokaryotic and eukaryotic organisms, and membrane metal transporters play essential roles in that regard. Dysfunction of metal transporters is detrimental to virtually every aspect of biological activities, and usually

associated with disease. Among these, the Nramp/Slc11 transmembrane proteins constitute a ubiquitous family of metal transporters important for host-microbe interactions and responsible for competitive acquisition for divalent cations. In spite of their wide taxonomic distribution, our current knowledge on these proteins is substantially limited, particularly concerning their 3D structure, subcellular localization, and mechanism of transport. Although current data support a three-dimensional model shared by other members of Nramp/Slc11 transporter family and other membrane cation-driven symporters, a crystal structure of Nramp that would provide high resolution details has not been resolved yet. A combination of both functional and structural approaches will be necessary to characterize the mechanistic aspects of Nramp proton/metal symport. Finally, the diversity and subcellular compartmentalization of Nramp isotypes/isoforms in both host and pathogen is far from being fully addressed. Thus, future studies focused on the above mentioned voids in our knowledge about structure and function(s) of Nramp should provide new insight into various aspects their role(s) in infectious disease, and reveal their potential as candidate drug targets.

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Abbreviations

Abbreviations used throughout the text are as follows: ABC, ATP-binding cassette; Abeta: amyloid beta-peptide; APX, ascorbate-dependent peroxidase; CALHM1, calcium homeostasis modulator 1; CDF: Cation diffusion facilitator; DFO, desferrioxamine; DMT1: divalent metal transporter 1; EST, expressed sequence tags; LD100, absolute lethal dose; MntH, H+-dependent Mn transporter; Nramp, natural resistance-associated macrophage protein; PAH, pulmonary arterial hypertension; PASMC, pulmonary artery smooth muscle cell; PS, photosynthesis system; ROS, reactive oxygen species; SERCA2a, sarcoplasmic/endoplasmic reticulum calcium ATPase; SLC11, solute carrier family 11; SOD, superoxidase dismutase; TC, transport classification; TMS, transmembrane segment.

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