Biochemical and Biophysical Research Communications 390 (2009) 136–141

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Functional assessment of Nramp-like metal transporters and manganese in *Caenorhabditis elegans*

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ARTICLE INFO

Article history:
Received 19 September 2009
Available online 26 September 2009

Keywords:
Caenorhabditis elegans Nramp homologs
Mn feeding
Pathogen
Caenorhabditis elegans intestine
Innate immunity
pmr-1

ABSTRACT

Nramp1 (natural resistance-associated macrophage protein-1) is a functionally conserved iron–manganese transporter in macrophages. Manganese (Mn), a superoxide scavenger, is required in trace amounts and functions as a cofactor for most antioxidants. Three Nramp homologs, *smf-1*, *smf-2*, and *smf-3*, have been identified thus far in the nematode *Caenorhabditis elegans*. A GFP promoter assay revealed largely intestinal expression of the *smf* genes from early embryonic through adult stages. In addition, *smf* deletion mutants showed increased sensitivity to excess Mn and mild sensitivity to EDTA. Interestingly, these *smf* deletion mutants demonstrated hypersensitivity to the pathogen *Staphylococcus aureus*, an effect that was rescued by Mn feeding or knockdown of the Golgi calcium/manganese ATPase, *pmr-1*, indicating that Mn uptake is essential for the innate immune system. This reversal of pathogen sensitivity by Mn feeding suggests a protective and therapeutic role of Mn in pathogen evasion systems. We propose that the *C. elegans* intestinal lumen may mimic the mammalian macrophage phagosome and thus could be a simple model for studying Mn-mediated innate immunity.

Introduction

Nramps (natural resistance-associated macrophage proteins) are a family of membrane proteins that facilitate the transport of heavy metal ions. Members of the Nramp family of protein transporters are evolutionarily conserved and are found in almost all organisms, from bacteria to man. In *Saccharomyces cerevisiae*, the three Nramp homologs Smf1p, Smf2p, and Sm3p, encoded by three distinct genes, are quite closely related [1]. Among the various divalent metals, these transporters are best known for manganese trafficking [2]. Increased levels or deprivation of intracellular manganese ions allows Smf1ps to shuttle between vacuoles and the plasma membrane for necessary degradation of the transporter or allows for manganese uptake from the environment, respectively [1].

The Nramps, in general, are well known to possess a significant conserved role in eukaryotic host defense. Besides being associated with innate resistance to certain bacterial infections, including several other infectious diseases in several vertebrate species, the Nramps in humans are known to play a crucial role in innate immunity [3]; therefore, they may be ascribed as the first line of intracellular defense against infection in humans. Likewise, mouse Nramp1 plays an important role in controlling infection by intracellular parasites and is exclusively expressed in monocytes/macrophages and polymorphonuclear leukocytes [4]. Nramp2, a more ubiquitously expressed transporter, acts as a divalent metal transporter capable of transporting iron, manganese, copper, zinc, cadmium, and lead [5].

Transition metals, including iron (Fe), zinc (Zn), and manganese (Mn), are essential cofactors for numerous proteins involved in vital functions, such as respiration, defense against oxidative stress, and cell division. Moreover, these metals are critical to both bacterial metabolism and virulence [6]. Depending on the microbe physiology, precise functions in survival, growth, and virulence may be affected by the amount of Mn ions available [7]. Transport of these essential metals out of macrophage phagosomes could exert pleiotropic effects on microbial metabolism and the capacity to perturb phagosome maturation.

In the nematode *Caenorhabditis elegans*, three Nramp type transporters, *smf-1*, *smf-2*, and *smf-3*, have been identified to date. *C. elegans*, a genetically tractable animal, is a facile and inexpensive model organism that has been used as a means for host–pathogen

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doi:10.1016/j.bbrc.2009.09.082

interaction studies, specifically to identify the host genes that interact with invading pathogens. In the present study we identified and characterized the Nramp homologs in *C. elegans*, namely, the three *smf* genes. The presence of these metal transporters in this model organism provides several advantages in determining their molecular identities. Mutants with deleted *smfs* were hypersensitive to excess Mn and displayed pathogen susceptibility, but subsequent Mn feeding facilitated the reversal of this phenotype. Our results describe the role of nematode *smfs* in Mn homeostasis and illustrate the function of Mn in the host–pathogen interaction. Recovery from pathogen sensitivity by Mn feeding suggests the protective and therapeutic potential of this metal in a pathogen evasion system. The *C. elegans* intestine may physiologically mimic the mammalian macrophage phagosome, functioning as a simple model for innate immune response studies.

Materials and methods

Caenorhabditis elegans strains, bacterial strains, and cosmid clones. *C. elegans* strains wild-type N2, *daf-16* (m26), *daf-2* (e1370), *smf-1* (ok1748), *smf-2* (gk133), and *smf-3* (ok1035) were obtained from the *Caenorhabditis elegans* Genetics Center (CGC) at the University of Minnesota, USA (Supplementary Material; Figure 1). Bacterial strains *Staphylococcus aureus* (KACC10196) and *Bacillus subtilis* (KACC10854) were obtained from the Korean Agricultural Culture Collection (KACC), Suwon, Korea. Cosmids K11G12 and Y69A2AR were obtained from A. Coulson at the Sanger Center, UK.

Construction of double mutant strains. Double mutants of *smf-1* (ok1748); *smf-3* (ok1035) and *smf-2* (gk133); and *smf-3* (ok1035) were constructed by standard genetic methods.

DNA constructs and microinjection. Approximately 1.5 kb of the 5′ upstream regions of *smf-1*, *smf-2*, and *smf-3* were amplified by PCR using cosmid K11G12 as a template for *smf-1* and *smf-2* and cosmid Y69A2AR as a template *for smf-3*. These PCR products were cloned into the promoterless *gfp* vector pPD95.77 to obtain *smf-1 pro::gfp*, *smf-2 pro::gfp*, and *smf-3 pro::gfp* (Supplementary Material; Figure 1). Microinjection was carried out as described [8].

Worm survival assays. For EDTA and manganese sensitivity experiments, L4 stage worms were transferred from normal NGM plates onto two different plates containing either 20 mM EDTA or 20 mM $MnCl_2$. Worms were incubated at 20 °C, and the number of dead animals was scored as described [9].

To examine pathogen susceptibility, *S. aureus* and *B. subtilis* strains were grown overnight at 37 °C in BHI liquid media. Bacterial lawns were prepared by spreading 10 μl of an original saturated bacterial culture diluted 10-fold onto 60-mm tissue culture plates containing solid BHI media (Difco). The plates were then incubated at 37 °C for 6 h, followed by 25 °C for 6 h. Worms were transferred to the plates and further incubated at 25 °C. The survival rate of each strain was scored after 3 days. Heat-killed *S. aureus* plates were prepared by heat treatment of the seeded plates at 65 °C for 2 h.

In the manganese (Mn) feeding experiment, L2 stage larvae were preincubated on NGM plates containing 1 mM $MnCl_2$ for 24 h and then washed on normal NGM plates for 24 h before being transferred to test plates.

In vitro transcription and RNA-mediated interference. Approximately 1.2 kb of *pmr-1* double stranded RNA was generated from yk218a11 cDNA clone as described earlier [9].

Statistical analysis. Data pertaining to survival rates of worms are presented as means ± SD. The survival rates of each animal strain were counted after 3 days following treatment. Forty animals were tested for each data point for a single experiment set, and each experiment was repeated six times. Data were evaluated by one-way analysis of variance (ANOVA) using the software Microcal Origin 6.0 (Microcal Software Inc., MA, USA). A level of $P < 0.05$ was considered the threshold for statistical significance between the control and the various experimental groups.

Results and discussion

The C. elegans smf genes

Thus far, three Nramp (natural resistance-associated macrophage protein)-like transporters, the *smf* genes, have been identified in the *C. elegans* database (see Wormbase; http://www.wormbase.org). The *smf* genes (*smf-1*, *smf-2*, and *smf-3*) have been physically mapped to Chromosome X (LGX) on cosmid K11G12 (*smf-1* and *smf-2*) and to Chromosome IV (LGIV) on cosmid Y69A2AR (*smf-3*). Both *smf-1* and *smf-2* have been identified as immediate neighboring genes on LGX (Supplementary Material; Figure 1). Alignment studies have shown that the worm Smf proteins show ~17–21% identity to yeast Smf proteins, ~41–48% identity to human Nramp1 or Nramp2, and ~42–59% identity to each other at the amino acid level (Supplementary Material; Figure 2).

Expression pattern of C. elegans smfs

In eukaryotes, the trafficking of manganese to antioxidant enzymes for enzyme activity is in part dependent on metal transporters like Nramps. In a step towards characterizing the physiological roles of the Nramp homologs in *C. elegans*, we first examined the temporal and spatial expression patterns of the *smf* genes. Transgenic worms containing the *smf-1 pro::gfp* or *smf-3 pro::gfp* construct demonstrated strong GFP expression at all stages of development, beginning as early as the comma stage embryo and continuing through larval and adult stages (Fig. 1A–J). Expression was spatially confined to intestinal cells, excretory cells, vulval epithelial cells, and neuronal cells. Fluorescence was largely observed at the apical ends of the adult and larval intestines (Fig. 1A, B, D, and G); in neuronal cells, particularly in the head neurons and hypodermis (Fig. 1E); in H-shaped excretory cells (Fig. 1F); and in vulval epithelial cells (Fig. 1H). Worms expressing the *smf-3 pro::gfp* construct exhibited distinct intestinal expression, which encompassed most of the intestine (Fig. 1I and J). In mammals, Nramp1s are abundantly expressed in the endosomal–lysosomal compartment of macrophages and are recruited to phagosomal membranes following phagocytosis [10]. In the present study, although *smf-1* and *smf-3* were expressed in various tissues and cell types, they were dominantly expressed in the intestines. Thus, they may have roles in the transport of metal ions in the intestine.

Under the control of the *smf-2* promoter, no expression of GFP was detected in the worms, indicating that the fragment selected as the putative promoter for *smf-2* may have lacked promoter activity. Besides being functionally related proteins, both *smf-1* and *smf*-2 physically mapped close together on Chromosome X (Supplementary Material; Figure 1) and therefore were found together in operons. *C. elegans* operons appear to be a means of coregulating functionally related proteins, similar to bacterial operons, and thus related genes do occur in operons [11].

SMF knockdown animals are sensitive to high concentrations of EDTA and Manganese

In order to investigate the *in vivo* function of the worm Smfs and to further study the transport activity of these metal transporters in response to metals, we first performed worm survival assays under high concentrations of EDTA (a metal ion chelator). Since SMFs are metal transporters, we reasoned that depletion of metals by a chelating agent could make the worms hypersensitive and have