Mycobacterium tuberculosis Expresses a Novel pH-dependent Divalent Cation Transporter Belonging to the Nramp Family

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Summary
Mammalian natural resistance–associated macrophage protein (Nramp) homologues are important determinants of susceptibility to infection by diverse intracellular pathogens including mycobacteria. Eukaryotic Nramp homologues transport divalent cations such as Fe^{2+}, Mn^{2+}, Zn^{2+}, and Cu^{2+}. Mycobacterium tuberculosis and M. bovis (bacillus Calmette-Guérin [BCG]) also encode an Nramp homologue (Mramp).

RNA encoding Mramp induces ~20-fold increases in Zn^{2+} and Fe^{2+} uptake when injected into Xenopus laevis oocytes. Transport is dependent on acidic extracellular pH and is maximal between pH 5.5 and 6.5. Mramp-mediated Zn^{2+} and Fe^{2+} transport is abolished by an excess of Mn^{2+} and Cu^{2+}, confirming that Mramp interacts with a broad range of divalent transition metal cations.

Using semiquantitative reverse transcription PCR, we show that Mramp mRNA levels in M. tuberculosis are upregulated in response to increases in ambient Fe^{2+} and Cu^{2+} between < 1 and 5 μM concentrations and that this upregulation occurs in parallel with mRNA for y39, a putative metal-transporting P-type ATPase. Using a quantitative ratiometric PCR technique, we demonstrate a fourfold decrease in Mramp/y39 mRNA ratios from organisms grown in 5–70 μM Cu^{2+}. M. bovis BCG cultured axenically and within THP-1 cells also expresses mRNA encoding Mramp.

Mramp exemplifies a novel prokaryotic class of metal ion transporter. Within phagosomes, M ramp and Nramp1 may compete for the same divalent cations, with implications for intracellular survival of mycobacteria.

Key words: bacillus Calmette-Guérin • Xenopus oocyte • metal ion • phagosome • intracellular pathogen

The macrophage vacuole is an especially hostile microenvironment for intracellular pathogens, as it is the arena in which multiple host defences operate. Many phylogenetically unrelated pathogens, including Mycobacteria, Salmonella, and Leishmania species, have successfully adapted to this niche (1, 2). A critical determinant of susceptibility to infection by these organisms is the natural resistance–associated macrophage protein (N ramp) family (3). N ramp homologues are phylogenetically ancient integral membrane proteins first identified in mice (4). A mutation in N ramp1 (originally designated Bgl2/Slh1/lyt) renders mice susceptible to uncontrolled proliferation of many organisms (M. bovis [BCG], M. avium, M. leprae).

Abbreviations used in this paper: BCG, bacillus Calmette-Guérin; 2-DG, 2-deoxy-[14C]glucose; M ramp, mycobacterial homologue of N ramp; N ramp, natural resistance–associated macrophage protein; ORF, open reading frame; RT, reverse transcription; SUM, standard uptake medium; TC, tandem competitive.

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sion is restricted to phagosomes of myelocytic cells (14). Some transition metals are also components of bacterial metalloenzymes (such as the superoxide dismutases and catalases) that protect bacteria against oxidative stresses encountered, for example, in phagosomes (15, 16).

We (17) and others (18, 19) have hypothesized that both mycobacteria and macrophages use N-ramp homologues to compete for intraphagosomal metal ions. We now provide evidence that M. ramp (the mycobacterial homologue of N-ramp) is a pH-dependent divalent cation transporter of broad specificity. M-ramp expression is modulated by variation in ambient Cu²⁺ and Fe²⁺ concentrations as well as being expressed in intracellular mycobacteria.

Materials and Methods

Isolation and Cloning of M-ramp Sequence. We used the previously identified M. leprae sequence encoding an N-ramp homologue to search the EMBL database using TBLASTN (20). PCR on genomic DNA from M. tuberculosis (H37Rv) and BCG using Pfu polymerase (Stratagene, Inc.) was carried out with primers designed to introduce BglII restriction sites and a strong eukaryotic Kozak consensus (TTGGTG

Expression of M-ramp in X enopus Oocytes and Fe²⁺ and Zn²⁺ Uptakes. Expression of M-ramp in X enopus Oocytes and Fe²⁺ and Zn²⁺ Uptakes was as described previously (22). Capped cRNA encoding M-ramp was transcribed (MEGAscript™ SP6; Ambion) from X bal.-linearized templates (pXM rmp and pXM pmr) and was injected into oocytes in mM CaCl₂, 2.4 mM NaHCO₃, 15 mM HEPES (pH 7.6), 0.3 mM Ca(NO₃)₂, 4H₂O, 0.41 mM CaCl₂, 6H₂O, 0.82 mM MgCl₂, 7H₂O, 10 μg/ml penicillin, 10 μg/ml streptomycin) at 19°C (23).

65Zn²⁺ uptake assays were performed on batches of 10–15 oocytes washed 4 times in freshly made up standard uptake medium (SUM) and sonicated (Rinco Ultrasonics) for 15 s (five 3-s bursts) to disaggregate bacterial clumps. The sonicate was added to macrophages (10 bacilli/macrophage) and incubated with SUM supplemented with Fe²⁺ (1 μM) and Cu²⁺ (0.5 μM) for 1 h at 37°C. Uptakes were performed with 55Fe²⁺ (1 μM) and Cu²⁺ (1 μM) or “high Cu” (5 μM), or “high Cu” (69.8 μM) were prepared by additionally supplementing aliquots of this medium with ferric ammonium citrate (16% Fe content) or CuCl₂. To ensure that concentrations of cations not being studied were above limiting concentrations, the Fe-modified medium were supplemented with Cu (1 μM), and Cu-modified media with Fe (4 μM). Concentrations of Fe and Cu in these media were verified by ferrozine assay (24) and atomic absorption spectrophotometry, respectively. All chemicals were obtained from Sigma-Aldrich.

Growth of M. tuberculosis in Varying Iron and Copper Concentrations. Starter cultures in Dubos broth supplemented with 10% human serum albumin (Difco) were initiated from glycerol stock and grown at 37°C to mid-log phase. These were inoculated (1:10) into low iron Sauton’s medium, grown for 1 wk (37°C, 5% CO₂, without shaking), and subcultured to ensure complete depletion of iron and copper. 10 ml of these cultures was inoculated into Fe/Cu-depleted Sauton’s medium (10 ml to 190 ml) supplemented to give low, medium, or high concentrations of iron or copper and grown for 5 wk.

Infection of M. rathbonei with BCG. As BCG encodes an M-ramp sequence (available from EMBL/GenBank/DDBJ under accession no. AJ005699) identical to that of M. tuberculosis, we used BCG as a model to examine the expression of M-ramp during intracellular infection. The human macrophage cell line, THP-1, was maintained as suspended cells and passaged at a density of 2 × 10⁶ cells/ml. Before infection with BCG, the cells were passaged at least three times in antibiotic-free RPMI 1640 (ICN Biochemicals) supplemented with heat-inactivated FCS (10%), grown to a density of 2.5 × 10⁶ cells/ml. Before infection with BCG, the cells were passaged at least three times in antibiotic-free RPMI 1640 (ICN Biochemicals) supplemented with heat-inactivated FCS (10%), grown to a density of 2.5 × 10⁶ cells/ml. Before infection with BCG, the cells were passaged at least three times in antibiotic-free RPMI 1640 (ICN Biochemicals) supplemented with heat-inactivated FCS (10%), grown to a density of 2.5 × 10⁶ cells/ml. Before infection with BCG, the cells were passaged at least three times in antibiotic-free RPMI 1640 (ICN Biochemicals) supplemented with heat-inactivated FCS (10%), grown to a density of 2.5 × 10⁶ cells/ml. Before infection with BCG, the cells were passaged at least three times in antibiotic-free RPMI 1640 (ICN Biochemicals) supplemented with heat-inactivated FCS (10%), grown to a density of 2.5 × 10⁶ cells/ml. Before infection with BCG, the cells were passaged at least three times in antibiotic-free RPMI 1640 (ICN Biochemicals) supplemented with heat-inactivated FCS (10%), grown to a density of 2.5 × 10⁶ cells/ml. Before infection with BCG, the cells were passaged at least three times in antibiotic-free RPMI 1640 (ICN Biochemicals) supplemented with heat-inactivated FCS (10%), grown to a density of 2.5 × 10⁶ cells/ml. Before infection with BCG, the cells were passaged at least three times in antibiotic-free RPMI 1640 (ICN Biochemicals) supplemented with heat-inactivated FCS (10%), grown to a density of 2.5 × 10⁶ cells/ml. Before infection with BCG, the cells were passed.
ings and microscopic examination of Ziehl-Nielsen-stained microphages. Macrophage viability (>90%) throughout these experiments was assessed by Trypan blue exclusion, with further details given in reference 25.

Recovery of RNA from Intraacellular BCG. After extensive washing of the macrophage monolayer, mycobacteria were recovered from differentially lysed THP-1 cells by the addition of 20 ml GTC solution (4 M guanidinium thiocyanate [GTC]; Fluka) containing 0.5% sodium N-lauroylsarcosine, 25 mM sodium citrate, pH 7, and 0.1 M 2-ME) to each flask (25). Total RNA was then extracted from the washed bacterial pellets as described previously (26).

Identification of ORF. Primers were designed so that the binding site is immediately upstream of the first ORF with antisense orientation in the same direction (30). A potential ribosome binding site for the fourth gene in this series. To ascertain if M ramp is cotranscribed with neighboring ORFs, we carried out RT-PCR on total RNA extracted from cultured H.37Rv. Using primers spanning the junction between the 3′ end of each fragment. These fragments were cloned in tandem into pGEM-T Easy (Promega). We chose “P”-type ATPase sequences from M. tuberculosis genomic DNA as template, and corresponded to the following fragments: 5′-ACGATCACCCATATACACAGG-3′ and 5′-CAGAAGAGCATTTACCAACC-3′. G- and C-primers were as follows: for Mramp, 5′-CTGGTTGC-3′ and 5′-GGACATTTGCAAGCG-3′; for y39, 5′-GTGGGGGCCCCTGAAGAAAGATTTCTGACGCGCA-3′ and 5′-CTTGCTGACACCAGCAGCTT-3′; for yhho, 5′-ACCGCTGGAATGGGTGATTCTGCAGAGCCAGCTTC-3′ and 5′-AACGAATTTCTTCGAGGCCTCAACCCAGCGC-3′.

Primers were designed such that the M ramp antisense primer overlapped with the yhho sense primer, and the yhho antisense primer overlapped with the y39 sense primer, giving a tandemly organized construct after synthesis by PCR (28). PCR cycle conditions, identical for each fragment, were as follows: 3 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 65°C, 2 min at 72°C, followed by a 4-min hold at 72°C. To discriminate competitor from target in tandem competitive (TC)-PCR reactions, a mutant asymmetrical KpnI restriction site in which the polar and nonpolar residues are segregated to opposite faces of the predicted topologies for yeast homologues, is shorter but exhibits a similar clustering of polar residues (31). The COOH terminus is also shorter and probably lacks the two final transmembrane segments predicted in some eukaryotic homologues. The amphiphilic properties of transmembrane segments M3, M5, and M9, in which the polar and nonpolar residues are segregated to opposite faces of the predicted α-helices (possibly forming a transmembrane channel), are also found in M ramp.

Sequence analysis of M ramp. A single N ramp homologue (designated M ramp) is located on cosmids MTY 21C12 (GenBank/EMBL/DDBJ accession no. Z95210; gene number Rv0924c) in a 5286-bp region containing 5 ORFs oriented in the same direction (30). A potential ribosome binding site is immediately upstream of the first ORF with M ramp as the fourth gene in this series. To ascertain if M ramp is cotranscribed with neighboring ORFs, we carried out RT-PCR on total RNA extracted from cultured H.37Rv using primers spanning the junction between the 3′ end of M ramp and the next ORF. A product of expected size (407 bp) was obtained (data not shown), indicating that M ramp is transcribed at least as a bicistronic operon.

Database searches failed to identify functionally characterized homologues of this second ORF. M ramp encodes a predicted 428-amino acid protein with a molecular mass of 44.9 kD and 10 transmembrane segments consistent with proposed topologies for yeast homologues (SNF1 and 2) (31). Compared with eukaryotic homologues, the hydrophilic NH2-terminal region of M ramp, like those of other prokaryotic N ramp homologues, is shorter but exhibits a similar clustering of polar residues (31). The COOH terminus is also shorter and probably lacks the two final transmembrane segments predicted in some eukaryotic homologues. The amphiphilic properties of transmembrane segments M3, M5, and M9, in which the polar and nonpolar residues are segregated to opposite faces of the predicted α-helices (possibly forming a transmembrane channel), are also found in M ramp.

Sequence analysis of M ramp confirms that certain amino acid residues are highly conserved among all members of the N ramp family (Fig. 1). M ramp sequences from M. tuberculosi and BCG are identical and are most closely related to other bacterial homologues (72, 4, 40, and 40% sequence identities with M. leprae, B. subtilis, and E. coli, respectively), whereas comparison with eukaryotic homologues gives overall amino acid identities of 21–24%. There

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Mramp Transports Cations

is an asymmetrical distribution of charged amino acid residues between the endo- and exofacial regions of Mramp. This is consistent with similar patterns of charge distribution observed in many integral membrane proteins (32).

Mramp Induces $^{65}$Zn$^{2+}$ Uptake in Oocytes. Initially we cloned Mramp into pGEM-T Easy after mutating the mycobacterial GTG start codon to ATG without strengthening the Kozak consensus sequence. Microinjection of oocytes with RNA made from this construct induced up to twofold increases in $^{55}$Fe$^{2+}$ and $^{65}$Zn$^{2+}$ uptake compared with water-injected oocytes (data not shown). To optimize Mramp expression in oocytes, we retained the modified start codon, introduced a strong Kozak consensus, and cloned Mramp into a vector containing flanking X. laevis 5' and 3' untranslated regions (see Materials and Methods).

In 10 independent experiments, RNA derived from this latter construct induced large increases (up to 22-fold) in the accumulation of $^{65}$Zn$^{2+}$ by Mramp-expressing oocytes compared with water-injected controls (P < 0.001). THT1 is the T. brucei hexose transporter.

$^{65}$Zn$^{2+}$ uptake in water-injected and THT1 (5 ng)–injected control oocytes. THT1 is the T. brucei hexose transporter. There was no significant difference in $^{65}$Zn$^{2+}$ uptake between water- and THT1-injected groups (P = 0.5). (C) 2-DOG uptake in M ramp (5 ng), THT1 (5 ng), and water-injected oocytes. THT1 induces significantly greater 2-DOG uptake than either M ramp (P < 0.002) or water (P < 0.002). There was no significant difference in uptakes between the water- and M ramp-injected oocytes. Displayed are mean values (± SE) of uptakes (10 oocytes per experimental condition).

Figure 2. $^{65}$Zn$^{2+}$ uptake by oocytes expressing M ramp. (A) Induction of $^{65}$Zn$^{2+}$ uptake by M ramp in Xenopus oocytes injected with R NA (5 ng in 50 nl) transcribed from pXmramp. The difference in $^{65}$Zn$^{2+}$ uptake between experimental and water (50 nl)–injected control oocytes is highly significant (P < 0.001). Inset shows a separate experiment in which $^{65}$Zn$^{2+}$ uptake in M ramp cr NA–injected oocytes was compared with antisense cr NA (pmar)–injected control oocytes (P = 0.025). (B)
Fig. 3. pH dependence of 65Zn²⁺ uptake induced by Mramp. 65Zn²⁺ uptake was assayed in Mramp-injected and control oocytes in media varying in pH (see Materials and Methods). The increase in 65Zn²⁺ uptake (Fold) is shown for Mramp-expressing oocytes compared with controls. Uptakes at pH 5.5 (P < 0.001) and pH 6.0 (P ≤ 0.009) were significantly greater than uptakes under remaining conditions. There were no significant differences in uptake at pH 5.0, 6.5, and 7.0 (P > 0.1). Displayed are mean values (± SE) of uptakes (10 oocytes per experimental condition).

Fig. 4. Substrate specificity for Mramp. (A) Abolition of 65Zn²⁺ uptake by Mn²⁺ in Mramp RNA-injected oocytes. Increase in uptake compared with control oocytes in the absence and presence of Mn²⁺ (10 mM as MnCl₂; P = 0.01). (B) Uptake of 55Fe²⁺ by Mramp RNA-injected oocytes compared with water-injected controls (P < 0.001). (C) Influence of divalent cation competitors on 55Fe²⁺ (100 μM) uptake by Mramp RNA-injected oocytes. Data from one experiment. Displayed are mean values (± SE) of uptakes (10 oocytes per experimental condition).

Figure 4.

compared with water-injected (Fig. 2 A) or Mramp antisense-injected controls (Fig. 2 A, inset). To confirm that these induced 65Zn²⁺ uptakes were specific to Mramp, we also examined the uptake of 65Zn²⁺ in oocytes injected with RNA made from the Trypanosoma brucei hexose transporter (THT1 [33]; Fig. 2 B). As expected, there was no increase in 65Zn²⁺ uptake associated with expression of THT1, confirming the requirement for Mramp to induce 65Zn²⁺ uptake. Conversely, we demonstrated 2'-deoxy-d[¹⁴C]-glucose (2-D-G) uptake by THT1 but not by Mramp (Fig. 2 C). To confirm that accumulation of 65Zn²⁺ continued beyond these experimental time points, we monitored 65Zn²⁺ uptake for up to 4 h. The increase in uptake of 65Zn²⁺ was linear during this period, which encompasses the uptake times of experiments shown (slope 3.8 ± 0.63, P < 0.001).

Mramp-induced 65Zn²⁺ uptake is pH dependent. In oocytes, translocation of divalent cations by DCT1 (the rat N ramp2 homologue) depends on cotransport of protons, with maximal activity of DCT1 at an extracellular pH of 5.5. To determine if cation transport by Mramp displays a similar pH dependence, we measured 65Zn²⁺ uptake by oocytes incubated in extracellular pH values between 5.0 and 7.0. In nine independent experiments, 65Zn²⁺ uptake by oocytes was confined to extracellular pH values between 5.5 and 6.5 (Fig. 3) and was completely abolished at pH 7 or 5.

Mramp has broad cation transport specificity. As a first step to determine the specificity of Mramp for divalent cations in the d-block series, we measured the uptake of 65Zn²⁺ in the presence of an excess of unlabeled Mn²⁺. Mn²⁺ (10 mM) completely abolished uptake of 65Zn²⁺ (Fig. 4 A), indicating that Mn²⁺ competes with 65Zn²⁺ for binding to or uptake by Mramp.

We next used 55Fe²⁺ as a permeant to investigate in detail the substrate specificity of Mramp for divalent cations. Mramp mediates large increases in the uptake of 55Fe²⁺ at pH 5.5 (up to 18-fold) above water-injected oocytes (Fig. 4 B). This induced 55Fe²⁺ uptake is abolished by Mn²⁺ and Cu²⁺ (Fig. 4 C). In contrast, Mg²⁺, a divalent cation that does not belong to the d-block series, enhanced 55Fe²⁺ accumulation by oocytes ninefold compared with Mn²⁺ uptake in its absence (Fig. 4 C).

M. tuberculosis and y39 expression are modulated by changes in ambient iron and copper concentrations. Mycobacterial growth varied in media containing different concentrations of Fe²⁺ and Cu²⁺. At relatively high concentrations (>45 μM), Fe²⁺ and Cu²⁺ inhibited bacterial growth by 29 and 38%, respectively (Fig. 5 B). Mramp mRNA transcript was detectable in bacteria grown at all Fe²⁺ and Cu²⁺ concentrations tested (Fig. 5 A), including a faint band in bacteria grown in Fe²⁺-depleted culture medium, which was quantifiable using the GDS 7600 system but is not visible on the photograph. mRNA for y39 was similarly detectable at all Fe²⁺ and Cu²⁺ concentrations. In contrast, mRNA for yhho gave much fainter bands in bacteria grown in high Fe²⁺ concentrations and medium and high Cu²⁺ concentrations, and was undetectable in other conditions. rRNA (16S) RT-PCR analysis of these templates confirmed that initial total RNA template quantities were comparable for all conditions (Fig. 5 A, bottom).

Semiquantitative analysis of PCR products from Mramp and y39 (a putative Ca²⁺-translocating P-type ATPase [17]) using identical template concentrations and PCR conditions showed large increases in mRNA for Mramp (~50-fold) as Fe²⁺ concentration increases from <1 to 48 μM (Fig. 5 A). As Cu²⁺ concentrations increase over a similar range, mRNA for Mramp increases ~10-fold, and is maximal at 5 μM Cu²⁺. There is less increase in mRNA for y39 under these conditions (~17- and 5-fold, respectively).

To investigate the regulation of Mramp and y39 transcription more precisely, we used a ratiometric PCR technique called TC-PCR to quantitate mRNA for Mramp in relation to y39 in M. tuberculosis cultured in media containing these different concentrations of Fe²⁺ and Cu²⁺. The mRNA ratios for Mramp/y39 fell fourfold (from 0.44 to 0.11) when Cu²⁺ concentrations increased from 5 to 70
Mramp Is Expressed by BCG in the Intracellular Environment. (A) RT-PCR for Mramp and y39 carried out on total RNA isolated from M. tuberculosis H37Rv cultured in media containing different Fe\(^{2+}\) and Cu\(^{2+}\) concentrations. Equal amounts of RNA template (30 ng) were used in each reaction (3 ng for 16S RNA experiments). Lane 1, low Fe\(^{2+}\) (<1 μM); lane 2, medium Fe\(^{2+}\) (4 μM); lane 3, high Fe\(^{2+}\) (48 μM); lane 4, low Cu\(^{2+}\) (<0.5 μM); lane 5, medium Cu\(^{2+}\) (5 μM); lane 6, high Cu\(^{2+}\) (70 μM). (B) Bacterial growth after 5 wk culture at each metal ion concentration. (C) Top panel, RT-PCR for Mramp on total RNA isolated from axenically cultured and intracellular BCG. Equal amounts of RNA template (135 ng) were used in each reaction. Lane 1, template from extracellular (axenically cultured) BCG; lane 2, template from intracellular BCG pretreated with RNAse A; lane 3, template from intracellular BCG; lane 4, template from intracellular BCG pretreated with RNAse A. Bottom panel, RT-PCR for rRNA (16S) using amounts of total RNA (165 ng)Fig. 5 C (bottom). This experiment was verified by RT-PCR analysis of rRNA (16S) (Fig. 5 C, bottom).

Discussion

Although approximately 1.7 billion people (one third of the world’s population) are infected with M. tuberculosis at any one time, it is striking that, in the absence of coinfection with HIV, fewer than 10% of these will develop active disease during their lifetimes (34). Host genetic factors such as polymorphisms in Nramp1 clearly influence susceptibility to infection or disease caused by Mycobacteria species (35). For example, tuberculosis in a tribally mixed Gambian population was associated with two pairs of Nramp1 polymorphisms (8), and in another study involving members of Chinese and Vietnamese families with leprosy, haplotypes associated with Nramp1-linked polymorphisms were observed to be distributed nonrandomly between affected and unaffected family members (9).

The Nramp family of proteins is highly conserved between bacteria and mammals, and two eukaryotic examples have been shown to transport divalent cations such as Fe\(^{2+}\) and Mn\(^{2+}\) (12, 36, 37). Nramp1 is likely to perform similar transport functions to Nramp2, which mediates pH-dependent Fe\(^{2+}\) uptake in heterologous expression studies and in vivo (12, 36). Defining the transport specificities for Nramp1 has proved difficult (38), and all studies characterizing Nramp homologues have been carried out exclusively on eukaryotic sequences. Recent studies in RAW 264.7 cells overexpressing Nramp1 suggest that Nramp1 does contribute to iron mobilization from vesicles (39). Nramp1 also circumvents maturation arrest of phagosomes containing live BCG, permitting the increase in acidification normally seen in phagosomes containing killed BCG or latex beads (40). These observations point to the possibility that competition for transition metal ions may be important in determining maturational dynamics of phagosomes as well as their lethality for certain intracellular pathogens.

We studied a mycobacterial homologue of the Nramp family because of its potential relevance to intracellular survival. Nramp homologues are found in M. tuberculosis, M. leprae, M. smegmatis, and BCG (7, 17). These homologues (called Mramp) have been suggested to mediate the uptake of cations such as Fe\(^{2+}\), Mn\(^{2+}\), and Zn\(^{2+}\), which may be important in defence by microbial superoxide dismutase against the macrophage respiratory burst (17, 18). Our studies now provide direct evidence for a function of Mramp as a transporter of Zn\(^{2+}\) and Fe\(^{2+}\).

Furthermore, the enhanced uptake of \(^{65}\)Zn\(^{2+}\) and \(^{55}\)Fe\(^{2+}\) induced in oocytes expressing Mramp is abrogated by an excess of Mn\(^{2+}\) and Cu\(^{2+}\), but not by unrelated divalent cations such as Mg\(^{2+}\), suggesting important interactions between Mramp and these transition elements. In spite of divergence in primary sequence between N ramp1, DCT1 (a rat intestinal homologue of N ramp2), and M ramp and their diverse phylogeny, all three sequences can mediate the uptake of Fe\(^{2+}\) into Xenopus oocytes (12). DCT1 transports other members of the transition metal series, and this broad

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specificity is also observed for M ramp. Therefore, M ramp represents a novel class of prokaryotic metal ion transporter with representatives in other bacteria such as E. coli and B. subtilis (Fig. 1).

We examined the pH dependence of cation transport by M ramp using $^{65}$Zn$^{2+}$ as a permeant, because at pH > 6.0 it is difficult to manipulate the equilibrium between Fe$^{2+}$ and Fe$^{3+}$ even in the presence of reducing agents such as ascorbic acid (41). There is a narrow range of extracellular acid pH values that allows M ramp-induced uptake of $^{65}$Zn$^{2+}$ by oocytes. This pH range (5.5–6.5) coincides with estimates of ambient pH in the microenvironment of intraphagosomal mycobacteria (42). This observation also provides evidence for the direction in which cation transport is likely to be taking place, namely from the relatively acidic phagosome into mycobacteria. By contrast, an uninfected phagolysosome (for example, one containing inert particles) has a significantly lower pH (<5.5 [42]), and would therefore be unlikely to allow efficient transport of divalent cations by M ramp.

To assess the expression of M ramp in M. tuberculosis cultured axenically, we applied a precise assay to quantitate mRNA for M ramp obtained from organisms grown in media containing defined Cu$^{2+}$ and Fe$^{2+}$ concentrations. These studies permitted assessment of the growth characteristics of bacteria as well as the relative expression of mRNA for M ramp compared with mRNA encoding a putative Ca$^{2+}$-transporting P-type ATPase (y39). M ramp is expressed poorly in bacteria grown in relatively Cu$^{2+}$- and Fe$^{2+}$-deficient media, and expression is enhanced at higher concentrations of these metal ions (≈5 μM). Similar patterns of mRNA expression are observed for the putative Ca$^{2+}$-transporting P-type ATPase (y39), but in contrast, mRNA encoding an atypical heavy metal-translocating P-type ATPase (yho) is barely detectable under any of the conditions tested (Fig. 5). This stimulation of expression of mRNA for M ramp and y39 at higher ambient concentrations of Cu$^{2+}$ and Fe$^{2+}$ is associated with retardation of bacterial growth by ~30%.

mRNA for M ramp encoded by BCG is clearly expressed in the intracellular environment (Fig. 5 C). We used BCG as a model for M. tuberculosis because M. tuberculosis is frequently cytopathic when cultured in THP1 cells, compromising yields of RNA. BCG is well recognized as a convenient model to study mycobacterial gene expression in these circumstances (25).

M ramp may act in concert with mechanisms inhibiting acidification of phagosomes to permit intracellular survival of mycobacteria. The deployment of N ramp1 in the host's phagosomal membrane is clearly important in defence against infection, as established by classical studies on the genetics of N ramp1. If N ramp1 also uses phagosomal protons to extrude cations, thereby competing with M ramp, the pH dependence of this phenomenon will be critical in establishing which of the two transporters (M ramp or N ramp1) functions most efficiently in the infected macrophage. Experiments to examine this hypothesis in greater detail can now be formulated on the basis of M ramp's function as defined by heterologous expression. M ramp is the first mycobacterial gene to be expressed in oocytes, exemplifying the utility of this system for the functional characterization of other prokaryotic transporters (E. coli glycero facilitator glpF, and E. coli water channel AqpZ [43]).

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