Changes of the phagosomal elemental concentrations by *Mycobacterium tuberculosis* Mramp

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Pathogenic mycobacteria survive within phagosomes which are thought to represent a nutrient-restricted environment. Divalent cation transporters of the Nramp family in phagosomes and mycobacteria (Mramp) may compete for metals that are crucial for bacterial survival. The elemental concentrations in phagosomes of macrophages infected with wild-type *Mycobacterium tuberculosis* (*M. tuberculosis* strain H37Rv) and a *M. tuberculosis* Mramp knockout mutant (Mramp-KO), derived from a clinical isolate isogenic to the strain MT103, were compared. Time points of 1 and 24 h after infection of mouse peritoneal macrophages (*bcg*) were compared in both cases. Increased concentrations of P, Ni and Zn and reduced Cl concentration in Mramp-KO after 1 h of infection were observed, compared to *M. tuberculosis* vacuoles. After 24 h of infection, significant differences in the P, Cl and Zn concentrations were still present. The Mramp-KO phagosome showed a significant increase of P, Ca, Mn, Fe and Zn concentrations between 1 and 24 h after infection, while the concentrations of K and Ni decreased. In the *M. tuberculosis* vacuole, the Fe concentration showed a similar increase, while the Cl concentration decreased. The fact that the concentration of several divalent cations increased in the Mramp-KO strain suggests that Mramp may have no impact on the import of these divalent cations into the mycobacterium, but may function as a cation efflux pump. The concordant increase of Fe concentrations within *M. tuberculosis*, as well as within the Mramp-KO vacuoles, implies that Mramp, in contrast to siderophores, might not be important for the attraction of Fe and its retention in phagosomes of unstimulated macrophages.

INTRODUCTION

Pathogenic mycobacteria such as *Mycobacterium tuberculosis* and *Mycobacterium avium* survive and replicate within the phagosome, which is thought to be a nutrient-restricted environment important to limit the nutritional needs of phagocytozed microbes. The regulation of the concentration of trace elements in the mycobacterial phagosome, especially of Fe, is known to be of crucial importance for the survival of intracellular organisms (De Voss et al., 2000).

The solute carrier family 11 member 1, Slc11a1, natural-resistance-associated macrophage protein 1 (Nramp1), is assumed to regulate the intraphagosomal replication of unrelated pathogens (mycobacteria, *Salmonella* and *Leishmania*) by controlling divalent cation concentrations at that site (Govoni & Gros, 1998). Polymorphisms in and around the *Nramp1* locus have been genetically linked to the susceptibility to acquire and develop tuberculosis (Awomoyi et al., 2002; Bellamy et al., 1998) and leprosy (Abel et al., 1998) in humans. Nramp1, localized to late endosomal/lysosomal membranes, is a divalent cation pump (Barton et al., 1999), but whether Nramp1 functions as an efflux pump or as an influx pump is an ongoing debate (Forbes & Gros, 2001; Gomes & Appelberg, 2002; Kuhn et al., 2001). Nramp1 may also act as a fusogen that promotes vesicle fusion, resulting in recruitment of vacuolar...
H\textsuperscript{+}-ATPase activity (Hackam et al., 1998). Therefore, it is possible that Nramp1 recruitment may increase the fusogenic properties of mycobacterial phagosomes (Forbes & Gros, 2001). Finally, Nramp2 has been implicated as a mediator of microtubule-dependant phagosome and/or lysosome transport (Tokuraku et al., 1998). Co-localization of mycobacterial phagosomes with the Nramp1 protein has only been shown in some, but not all M. avium-containing phagosomes (Searle et al., 1998), but it clearly affects intracellular survival of M. avium in bone marrow-derived mouse macrophages (Frehel et al., 2002).

In mammals, a second homologue, Nramp2 (DCT1/DMT1), is expressed in many different cells in contrast to the macrophage-/monocyte-specific Nramp1. In intestine and other tissues, Nramp2 is involved in Fe transport (Gruenheid et al., 1999); however, studies in Xenopus laevis oocytes have demonstrated that Nramp2 might transport a broad range of divalent metals (Gunshin et al., 1997; Picard et al., 2000; Tandy et al., 2000). In macrophages, Nramp2 is primarily detected in recycling endosomes and also, to a lesser extent, at the plasma membrane, co-localizing with transferrin. It suggests a role for transferrin receptor. We part, through the activity of the transferrin receptor. We have also shown that siderophore production is necessary to retain Fe in the mycobacterial phagosome (Maser et al., 2003; Wagner et al., 2005). In the present study, we have used a hard X-ray microprobe to compare the elemental concentrations in phagosomes infected with wild-type M. tuberculosis and an Mramp knockout mutant (Mramp-KO). Our data showed that, during infection, the concentration of several divalent cations in the phagosome is increased by the absence of the Mramp gene, indicating that Mramp may function as a cation exporter. The concentration of Fe, however, appears not to be affected.

**METHODS**

**Mycobacteria and culture conditions.** Mycobacterium tuberculosis H37Rv was purchased from ATCC and the Mramp mutant of M. tuberculosis (a clinical isolate isogenic to strain MT103) was created as described previously (Boechat et al., 2002). The bacteria were cultured as reported previously (Boechat et al., 2002) in Middlebrook 7H9 broth (Difco) or on Middlebrook 7H11 agar (Difco).

**Comparison between H37Rv and MT103 strains.** To determine whether H37Rv and MT103 strains replicated similarly in macrophages, mouse peritoneal macrophages from C57BL/6 (bcg) mice were isolated as described previously (Bermudez et al., 1997). Macrophages in culture with RPMI 1640 supplemented with 10% fetal bovine serum (FBS; Sigma) for 2 days were infected with either MT103 or H37Rv at a ratio of 10 bacteria to 1 macrophage. Uptake was allowed to occur for 1 h and then extracellular bacteria were removed by washing twice with Hank’s Balanced Salt Solution (HBSS). Macrophage monolayers were lysed up to 7 days after infection to determine the number of intracellular bacteria, as described by Wagner et al. (2002). Supernatants of infected and control macrophages were also collected and assayed for both TNF-α and p70 interleukin-12 (IL-12) by using commercial kits (Biosource International) according to the manufacturer’s instructions. The assays had a sensitivity of 5 and 4 pg ml\textsuperscript{-1}, respectively.

**Specimen preparation.** Mouse peritoneal macrophages from C57BL/6 (bcg) mice (10\textsuperscript{6} cells) were seeded on 200-mesh, Formvar-coated London finder gold grids (Electron Microscopy Sciences) and cultured for 24 h in RPMI 1640 supplemented with 10% FBS. The monolayers were infected with either M. tuberculosis (strain H37Rv) or the Mramp-KO strain of M. tuberculosis for 1 h with an approximate ratio of 10 bacteria per macrophage. After 1 h of infection, the macrophages were washed twice with HBSS to remove the remaining extracellular bacteria, then either fixed with 1% paraformaldehyde (pH 7-2) for 30 min or kept for 24 h in RPMI 1640 supplemented with 10% FBS before washing and fixation. This yielded time points of 1 and 24 h after infection. The remaining paraformaldehyde was removed by washing twice with HBSS. The grids were subsequently rinsed briefly with sterile water, air-dried and then kept at room temperature in viewing chambers for the microscopic identification of bacteria or phagosomes by phase microscopy using a Nikon microscope with a PlanApo 60 x /1.3 oil immersion lens.

**Hard X-ray microprobe and allocation of the phagosome.** A hard X-ray microprobe (Cai et al., 2000) was used to collect...
quantitative maps of the elemental distribution in the macrophage and the phagosome. Details regarding data collection have been described previously (Maser et al., 2003). Prior to X-ray imaging, the specimen was previewed by an optical microscope and x/y coordinates for each phagosome were recorded. The specimen was then placed in the hard X-ray microprobe. An incident photon energy of 10 keV was chosen, which allowed measurement of Zn and lighter elements. Each phagosome, based on the previously measured coordinates, was positioned into the focused X-ray spot and raster-scanned. For all Mramp-KO specimens, a full X-ray fluorescence spectrum was acquired at each pixel of the scan. For M. tuberculosis specimens, 10 elemental channels were collected as reported previously (Maser et al., 2003; Wagner et al., 2005).

Data analysis. Elemental maps for Mramp-KO specimens were extracted from the X-ray fluorescence spectra using the software package MAPS (Vogt, 2003; Vogt et al., 2003). Quantification was achieved by measuring X-ray fluorescence from NIST thin-film standards NBS183 and NBS1833 (National Bureau of Standards, Gaithersburg, USA) prior to, during and after each experiment. A calibration curve was extracted from the calibration standards and subsequently applied to quantify all elements from Na to Zn. The calibration curve was based on direct quantification of elements Si, Ca, K, Ti, V, Mn, Co, Cu, Fe and Zn, interpolation for elements P, S, Cl and Ni, and extrapolation for Na and Mg. For improved accuracy, the mean elemental content for each mycobacterium was calculated in the following process: in each scan, both the area of the mycobacterium and an area representative of the local cellular background were identified. From fitting these mean spectra and comparison to the calibration curve, raw elemental concentrations were calculated for each mycobacterium and a defined background region. The background signal was estimated by determining the content of the cytoplasm in the very proximity (within 400–600 nm) of the phagosome. We used this signal as a representation of the content of the cytoplasm above and below the phagosome. The net elemental content of each bacterium was then calculated by subtraction of the elemental background of the cell from the raw elemental content of the bacterium. The resulting data were obtained as area density and expressed as μg cm⁻². These values can be calculated as mM l⁻¹ or μM l⁻¹ by using the molar mass of the respective elements and the thickness of the mycobacteria, which we assumed to be 1 μm. At fixed excitation energy, the sensitivity of the measurements is reduced for lighter elements due to both smaller X-ray fluorescence yield and smaller absorption cross-section (Jenkins et al., 1995; Thompson et al., 2001). Therefore, only relatively large concentrations of P, S and Cl can be detected. Sensitivity to Na and Mg is even smaller. Sensitivity to trace metals such as Fe, Mn, Cu and Zn, however, is very high.

Previously data collection on M. tuberculosis phagosomes (Wagner et al., 2005) did not allow us to save the full spectra at each pixel. This led to a reduction in sensitivity to Ca in the presence of high K concentrations and did not allow quantification of P, S, Cl and Ni as area density. The concentration of these elements is expressed in normalized counts (photon counts per second of data acquisition time and synchrotron current) to allow comparison of the data.

Statistical analysis of observed elemental changes was performed by comparing the concentration of the respective elements at a specific time point in the different groups (wild-type versus Mramp-KO) and within a group (1 h versus 24 h) using Student’s t-test. A P-value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

The concentration of elements in the phagosome of bacteria ingested by macrophages is thought to be tightly regulated as part of the mechanism of the macrophage to impair bacteria. An example is the delivery of Fe via the transferrin receptor to the phagosome, which is thought to be used to damage bacterial DNA via Fenton catalysis of toxic reactive oxygen species (Henle & Linn, 1997; Kuhn et al., 2001; Wagner et al., 2005; Zhong et al., 2001). At the same time, the macrophage vacuole is thought to reduce the amount of nutrients available to the bacterium in an attempt to impair it by starvation. Possible candidates for the transport of divalent cations from the endosomal system into the cell cytosol are members of the divalent cation transport family of proteins, Nramp1 and Nramp2, that are recruited to phagosomes at different stages of the endosomal continuum (Gruenheid et al., 1999; Searle et al., 1998). Intracellular bacteria, like pathogenic mycobacteria, however, have developed strategies to counteract these lines of attack, such as producing superoxide dismutases or catalases to detoxify reactive oxygen species (Bunting et al., 1998; Escuyer et al., 1996; Piddington et al., 2001), or producing siderophores to acquire Fe (De Voss et al., 2000). Also, the production of metal ion transporters by mycobacteria, like Mramp and heavy metal ‘P’-type ATPases, has been implicated in the competition against the deprivation of metal cations (Agranoff et al., 1999; Agranoff & Krishna, 1998). Mramp mRNA has been shown to be produced by M. bovis BCG after 1 day of infection of THP-1 cells (Agranoff et al., 1999), suggesting a function at that time. Therefore, we conducted a study to directly measure and quantify the concentration of metals in vacuoles of mouse macrophages containing M. tuberculosis bacteria in which the Mramp gene had been knocked out. The hard X-ray microprobe beamline was calibrated using NIST thin-film standards, which yields a variability of quantification between different experimental runs of less than 10% (data not shown).

In our study we used macrophages from C57BL/6 (bcg²) mice. The Mramp mutant strain displayed no impairment of growth or survival in Nramp1⁺/⁺ and Nramp1⁻/⁻ congenic murine macrophage cell lines (Boechat et al., 2002). Since our study focused on the function of the Mramp gene in vitro, and since the presence or absence of the Nramp1 gene does not affect the virulence of the Mramp mutant strain, we did not examine the interaction of Mramp with the Nramp proteins in cells expressing a functioning Nramp1 protein.

Evaluation of the ability of H37Rv and MT103 to replicate in mouse macrophages showed that the two bacteria have comparable behaviour (Fig. 1). In addition, the kinetics of induction of TNF-α and IL-12 production were similar for both bacteria (Table 1). Based on these results, we decided to use strain H37Rv as the wild-type control because the availability of hard X-ray microscopy data for this strain is significantly greater.

Experiments were carried out in macrophages infected with the Mramp mutant at 1 and 24 h following infection. We used the same time points as in previous experiments
on *M. tuberculosis*, in which we had observed significant differences in the concentration of several elements in different mycobacterial species (Maser et al., 2003; Wagner et al., 2005). Data for the *M. tuberculosis* wild-type from that study were used (six and seven mapped phagosomes for the 1 and 24 h time points, respectively) as a comparison for the *Mramp* mutant. In this mutant, nine different phagosomes in different macrophages from two different preparations were mapped for each time point. Fig. 2 shows an optical differential interference contrast image of an infected macrophage (Fig. 2a), the elemental distribution of Cl in the macrophage (Fig. 2b) and elemental distributions and X-ray fluorescence spectra of one individual phagosome (Fig. 2c–e). Table 2 lists the different concentrations of the elements in the vacuoles of these mycobacteria and Fig. 3(a–e) shows a schematic representation of these results for the cations of Ca, Zn, Ni, Mn and Fe. Following 1 h of infection, concentrations of P, Ni and Zn were significantly increased in the *Mramp*-KO strain compared to the wild-type strain, while the Cl concentration decreased. At the 24 h time point, differences in the concentrations of P, Cl and Zn were still observed when compared to the concentrations in the wild-type phagosome after 1 day of infection. At this time point, concentrations of Ca were also significantly higher, while the lower K concentration seen in the *Mramp*-KO strain was close to being significant (*P*=0·057).

When the comparison was made for the *Mramp*-KO strain after 1 and 24 h of infection, an increase in Ca, Mn, Fe, Zn and P concentrations was measured, together with a decrease in K and Ni concentrations. The decrease in the Cu concentration over the first day of infection was not significant (*P*=0·07). We observed a high variation in the K concentration in both wild-type and mutant phagosomes, similar to variances found in extracellular *M. avium* (unpublished data). It is possible that these variances represent different stages of mycobacterial development, asynchrony in the individual phases of bacterial invasion, or surviving and dead bacteria.

**Mramp and the phagosomal Fe concentration**

An important finding of our study was a concordant behaviour of the Fe concentration in the wild-type and *Mramp*-KO strains: in both the Fe concentration increased dramatically over time. The increase of Fe concentration in *M. tuberculosis* and the *Mramp*-KO vacuoles implies that Mramp, in contrast to siderophores, is not important for attraction and retention of Fe in phagosomes of unstimulated macrophages. This observation is in agreement with previous findings showing that the virulence of the *Mramp*-KO strain is not affected in mice (Boechat et al., 2002; Domenech et al., 2002). In addition, this finding supports the concept that the Fe concentration in the mycobacterial vacuole is important for intracellular survival (De Voss et al., 2000; Maser et al., 2003; Wagner et al., 2005).

The hard X-ray microprobe is capable of discerning very small amounts of elements, in the order of 10^{-16} g Zn, for example, inside the cell. However, current X-ray optics do not provide the spatial resolution required to discriminate between the vacuole and the bacterium. Since the vacuole membrane of the phagosome is very close to the outermost structure of the bacterium, we assume that most of the elements measured are located inside the bacterium. This would imply that the bacterium can somehow model the contents of the phagosome by importing and exporting elements. In fact, assuming that Mramp would transport specific cations within a phagosome during infection of macrophages into the mycobacterium, the concentration of this cation in the phagosome would be low, and a large portion of the cation would be inside the bacterium; the opposite would occur with the mutant. In contrast, if one assumes that Mramp would not transport a specific cation into the mycobacterium, the concentration of this cation in the phagosome would be high initially, but low after some time due to transport to the extraphagosomal environment. The concentration in the bacterium would

**Table 1. Production of TNF-α and IL-12 upon macrophage infection with *M. tuberculosis* strains H37Rv and MT103**

Data are based on 10^5 macrophages per monolayer. The values (pg ml^{-1}) are the means ± SD of two experiments.

<table>
<thead>
<tr>
<th>Strain</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TNF-α</td>
<td>IL-12</td>
</tr>
<tr>
<td>None</td>
<td>Undetectable</td>
<td>Undetectable</td>
</tr>
<tr>
<td>H37Rv</td>
<td>205 ± 24</td>
<td>126 ± 35</td>
</tr>
<tr>
<td>MT103</td>
<td>236 ± 29</td>
<td>118 ± 22</td>
</tr>
</tbody>
</table>

![Graph](image_url)

**Fig. 1.** Comparison between the ability of the *M. tuberculosis* strains H37Rv (circles) and MT103 (triangles) to replicate in C57Bl/6 mouse macrophages.
be low, as observed in vacuoles with *M. smegmatis*, a bacterium that does not retain Fe within the vacuole (Maser et al., 2003) (Fig. 4).

**Mramp – a divalent cation transporter?**

Our data do not provide conclusive evidence that the *Mramp* gene functions as a divalent cation transporter in the mycobacterium. The knockout of the *Mramp* gene leads to an increase of the phagosomal concentration of several divalent cations, like Ni and Zn, rather than a decrease after the first hour of infection. After 1 day of infection, the Ca and Mn concentration had increased, while the Zn concentration was further increased. This implies a transport from the cytoplasm to the vacuole and accumulation of these cations in the Mramp-KO strain that does not occur in the wild-type. The decrease in the Ni concentration in the Mramp-KO strain after 24 h suggests that Ni had been transported from the vacuole to the cytoplasm by other transporters. Previous transport studies have shown that the Mramp protein transports divalent cations into *Xenopus laevis* oocytes (Agranoff...
Table 2. Intravacuolar concentrations of single elements

Macrophages were infected with M. tuberculosis H37Rv (wild-type) and Mramp-KO for 1 or 24 h. Data for the different elements were acquired simultaneously for each phagosome; the number (n) of measurements is therefore the same for the different elements as given at the bottom of each column. Mean ± SEM are shown. Elements for which standards are available are expressed and mmol l⁻¹ or as μmol l⁻¹, assuming a mean mycobacterial thickness of 1 μm. For previously measured data of M. tuberculosis, no standard was available for P, S, Cl and Ni. All data for these elements are therefore expressed as counts per second of data acquisition time and synchrotron current (counts s⁻¹ A⁻¹). The Ca concentration for the 1 h time point of M. tuberculosis was below the sensitivity of the method for each measured phagosome (see Methods).

<table>
<thead>
<tr>
<th>Element (unit)</th>
<th>Wild-type 1 h</th>
<th>Mramp-KO 1 h</th>
<th>Wild-type 24 h</th>
<th>Mramp-KO 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>P (counts s⁻¹ A⁻¹)</td>
<td>7.05 ± 1.66</td>
<td>17.7 ± 2.08*</td>
<td>7.13 ± 1.36</td>
<td>39.5 ± 6.22*</td>
</tr>
<tr>
<td>S (counts s⁻¹ A⁻¹)</td>
<td>36.9 ± 5.53</td>
<td>26.7 ± 2.55</td>
<td>23.7 ± 3.90</td>
<td>58.0 ± 29.7</td>
</tr>
<tr>
<td>Cl (counts s⁻¹ A⁻¹)</td>
<td>40.0 ± 56.1‡</td>
<td>68.7 ± 10.4*</td>
<td>197 ± 62.1</td>
<td>47.4 ± 8.29*</td>
</tr>
<tr>
<td>K (mmol l⁻¹)</td>
<td>19.5 ± 16.9</td>
<td>46.8 ± 6.09‡</td>
<td>51.0 ± 28.6</td>
<td>3.3 ± 0.43‡</td>
</tr>
<tr>
<td>Ca (mmol l⁻¹)</td>
<td>0.00 ± 0.00</td>
<td>0.35 ± 0.17†</td>
<td>0.25 ± 0.17</td>
<td>7.49 ± 0.95*</td>
</tr>
<tr>
<td>Mn (μmol l⁻¹)</td>
<td>14.7 ± 5.20</td>
<td>16.0 ± 3.80†</td>
<td>16.9 ± 10.9</td>
<td>38.4 ± 8.43</td>
</tr>
<tr>
<td>Fe (μmol l⁻¹)</td>
<td>135 ± 41.8‡</td>
<td>194 ± 6.4†</td>
<td>2680 ± 974</td>
<td>2913 ± 820</td>
</tr>
<tr>
<td>Ni (counts s⁻¹ A⁻¹)</td>
<td>0.15 ± 0.058</td>
<td>5.4 ± 0.64‡</td>
<td>0.07 ± 0.048‖</td>
<td>0.7 ± 0.27</td>
</tr>
<tr>
<td>Cu (μmol l⁻¹)</td>
<td>426 ± 393</td>
<td>87 ± 16.4§</td>
<td>25 ± 9.52</td>
<td>49 ± 10.5</td>
</tr>
<tr>
<td>Zn (μmol l⁻¹)</td>
<td>37.8 ± 25.2</td>
<td>504.5 ± 61.1†</td>
<td>456.8 ± 271</td>
<td>1057 ± 118*</td>
</tr>
</tbody>
</table>

n = 7, n = 9, n = 6, n = 9

*P < 0.05 versus wild-type (same time point).
†P < 0.05 versus Mramp-KO, 24 h.
‡P < 0.05 versus wild-type, 24 h.
§P = 0.057 versus wild-type, 24 h.
‖P = 0.07 versus Mramp-KO, 24 h.

et al., 1999). This does not exclude the possibility that the Mramp protein in the mycobacterial membrane is in an orientation that would lead to an export of these cations, like Ca, Mn, Ni or Zn, out of the mycobacterium. A similar debate is ongoing about the direction of the Nramp1 cation transport in the phagosomal membrane (Forbes & Gros, 2001; Gomes & Appelberg, 2002; Kuhn et al., 2001). In this case, the missing Mramp protein in knockout mutants would lead to an accumulation of these cations, which could have been transported to the phagosome and into the mycobacterium by other cation transporters. In the wild-type, with lower concentrations of Ca, Mn, Ni and Zn, the Mramp protein could have transported these cations out of the mycobacterium and other transport proteins could have exported these from the phagosome to the extraphagosomal environment (Fig. 3).

In Salmonella, Mramp functions mainly as an Mn²⁺ uptake pump (Kehres et al., 2000). It is expressed after invasion of macrophages and induced by hydrogen peroxide (which kills the knockout mutant strain more effectively). However, similar to the mycobacterial Mramp, the Salmonella Mramp does not affect invasion or survival of the bacterium in macrophages in vitro and only slightly impacts on virulence in a mouse model of infection (Kehres et al., 2000, 2002). In our study, we did not observe a decreased Mn uptake in comparison to the wild-type in mouse peritoneal macrophages. However, the Mn concentration was increased in the vacuole of the Mramp-KO strain after 1 day of infection compared to after 1 h of infection, which was not observed in the wild-type. A similar increase in the Mramp-KO strain compared to the wild-type was also seen for Ca and Zn concentrations, implying an increased delivery of these cations to the phagosome infected with the Mramp-KO strain over time. Again, in the wild-type with lower Ca, Mn and Zn concentrations, Mramp could export these cations during the process of infection, thus preventing this accumulation seen in the Mramp-KO strain (Fig. 3). The implication of these regulatory mechanisms is unclear at the moment. The future identification of specific mutants will provide the tools to further dissect such mechanisms.

Mramp – correlation between Cl and pH?

We found a higher Cl concentration in the wild-type phagosome at both time points compared to the mutant, with the largest concentration after 1 h of infection for the
Fig. 3. Schematic representation of the results for the concentration of the cations of Ca (a), Zn (b), Ni (c), Mn (d) and Fe (e). The dots schematically represent the measured concentration of the respective single element (E). Since current X-ray optics do not provide the spatial resolution required to discriminate between the vacuole and the bacterium, the distribution of the concentration of an element between mycobacterium (M) and phagosome (P), and cytoplasm (C) is a presumption (see Fig. 4). T, Mramp transporter. (a) The Ca concentration is low in the wild-type and the Mramp-KO strain after 1 h of infection. It increases significantly after 24 h only in the knockout mutant. (b) The Zn concentration is low in the wild-type and increases significantly after 24 h of infection. In the knockout mutant it is already increased during the first hour and increases further after 24 h. (c) In the Mramp-KO strain, the Ni concentration strongly increased during the first hour and decreased after 24 h, while it stayed low in the wild-type. (d) In the knockout mutant, the Mn concentration stayed low in the wild-type and increased after 24 h in the knockout mutant. (e) The Fe concentration increased dramatically over time in both wild-type and Mramp-KO strains.
wild-type and a significantly reduced concentration after 24 h. A recent paper in which the Cl concentration was determined in early and late endosomes using fluorescent ligands suggests that Cl is the principal counter ion accompanying endosomal and Golgi compartment acidification (Sonawane & Verkman, 2003), implying that Cl influx is paralleled by proton influx, and a high Cl concentration may represent a low pH. In mycobacterial phagosomes, a considerable heterogeneity of pH among different vesicles, especially in the first hours of infection, and a detectable initial drop in pH, followed by a recovery and equilibration at pH 6.3–6.5, have been described (Deretic & Fratti, 1999). We speculate that the high initial Cl concentration and the following decrease in the wild-type may reflect this initial drop and later increase of the pH. In the Mramp-KO strain, the accumulation of Cl, especially in the first hours of infection, however, was not observed in this study. The function of Mramp is, similar to other members of this transporter family, supposed to be proton-dependent (Domenech et al., 2002), implying that Mramp is, as suggested for Nramp1 (Mulero et al., 2002), a divalent cation-proton antiporter and a cation efflux pump (as suggested by our data), it is tempting to speculate that the knockout of this transporter leads to less accumulation of protons in the mycobacterium and concomitantly to a lower Cl concentration. This hypothesis, as well as the question whether the decreased K concentration, seen in the Mramp-KO strain after 24 h of infection, is related to this phenomenon or to the missing efflux of divalent cations, need to be investigated in subsequent, specifically designed experiments.

In conclusion, the concentration of several divalent cations increased in the Mramp-KO vacuole within 1 h and over the 1 day post-infection period, indicating that Mramp may not import divalent cations into the mycobacterium, but rather may function as a cation efflux pump. The concordant increase of Fe concentrations in *M. tuberculosis* and the Mramp-KO vacuoles implies that Mramp, in contrast to siderophores, is not important for the attraction of Fe and its retention in phagosomes of unstimulated macrophages.

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