**Nramp1 Modifies the Fusion of Salmonella typhimurium-containing Vacuoles with Cellular Endomembranes in Macrophages**

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Salmonella survive and replicate within mammalian cells by becoming sequestered within specialized membrane-bound vacuoles inaccessible to the host defense mechanisms. Delayed acidification of the vacuole and its incomplete fusion with lysosomes have been implicated in intracellular Salmonella survival. Nramp1 confers macrophages resistance to a variety of intracellular pathogens, including Salmonella, but its precise mode of action is not understood. We investigated whether Nramp1 affects the maturation and acidification of Salmonella-containing vacuoles (SCV). A mouse-derived macrophage line (RAW/Nramp1−/−) devoid of Nramp1 and therefore susceptible to infection was compared with isogenic clones stably transfected with Nramp1 (RAW/Nramp1+). Intravacuolar pH, measured in situ, was similar in Nramp1-expressing and -deficient cells. SCV acquired LAMP1 and fused with preloaded fluid-phase markers in both cell types. In contrast, all intracellular Salmonella acquired LAMP1 and segregated into a characteristic SCV.

Infectious organisms are generally eliminated from the host, at least in part, by the innate immune system. Phagocytes ingest the microorganisms into a membrane-bound compartment or phagosome, which matures by sequential fusion with a series of endomembrane compartments (11). The resulting changes in the composition and luminal pH confer to the phagosome its characteristic microbicidal properties. To survive intracellularly, Salmonella evades the hostile environment of the phagosome by subverting the maturation process. Instead of undergoing the customary fusion with the endosomal and lysosomal compartments, yielding a phagolysosome, Salmonella generates a unique compartment designated the Salmonella-containing vacuole (SCV) that ultimately segregates itself from the endocytic pathway (12). Survival of bacteria within the SCV has been attributed to the incomplete fusion of the latter with the pre-lysosomal and lysosomal compartments (12, 13), which is accompanied by delayed and/or reduced luminal acidification (14).

Not all individuals are equally susceptible to Salmonella infection. This is attributed in some instances to the expression of host gene products that confer natural resistance to the bacteria (15–17). One such resistance factor is natural resistance-associated macrophage protein 1 (Nramp1), an integral membrane protein expressed in lysosomal compartments of macrophages (18, 19). The mechanism whereby Nramp1 confers to the host resistance to infection is incompletely understood. Following phagocytosis, Nramp1 reaches the phagosome membrane (18), where it has been suggested to prevent the replication of bacteria by limiting the availability of luminal divalent cations (20, 21). It is unclear, however, whether and how these putative alterations in divalent cation content modify the ability of the bacteria to evade phagosomal maturation and segregate into a characteristic SCV.
The purpose of this study was to determine whether the presence of Nramp1 alters the intracellular fate of Salmonella in cultured and in primary murine macrophages. Maturaion of the SCV was monitored using markers of the compartments of the endocytic pathway and measuring intravacular pH by digital imaging. Our results suggest that macrophages expressing the Nramp1 protein counteract the ability of Salmonella to become secluding in a compartment that limits access of bactericidal agents. We propose that Nramp1 facilitates bacterial killing by altering the pattern of vacuole fusion with endomembranes.

**EXPERIMENTAL PROCEDURES**

**Materials**—Rabbit antibody to M6PR (cation-independent M6PR300 mannos 6-phosphate receptor) was the generous gift from Drs. S. Honing and K. von Figura (University of Göttingen, Göttingen, Germany). ID4B monoclonal anti-mouse LAMP1 antibodies were from the Developmental Studies Hybridoma Bank. Anti-c-Myc antibody was from Santa Cruz Biotechnology. Rabbit polyclonal and mouse monoclonal anti-S. typhimurium LPS antibodies were from Difco Laboratories and from Advanced Immunochemical, respectively. Cy3-labeled secondary antibodies were from Jackson Immunoresearch. Secondary antibodies labeled with Alexa Fluor 350 or 488, fluorescein 5-isothiocyanate (FITC) and tetramethylrhodamine dextran (10,000 kDa) were purchased from Molecular Probes (Eugene, OR). Tissue culture supplies were obtained from CellGro. All other reagents were obtained from Sigma, unless otherwise stated.

**Cell Culture**—RAW264.7 cells are immortalized monocye/macrophages isolated from BALB/c mice that express Nramp1G169/G169 and are therefore Nramp1-defective and susceptible to invasion and replication of intracellular pathogens. To reflect the lack of functional Nramp1, these cells are called RAW/Nramp1-1 hereafter. Stable transfection of c-Myc-tagged Nramp1 into RAW264.7 macrophages yielded RAW/Nramp1-1 cells. The expression of Nramp1 in these clones overcomes their innate susceptibility to infection with Salmonella (22). Cells were maintained in Dulbecco’s modified Eagle’s medium (high glucose formulation supplemented with 10% fetal bovine serum, 200 mM L-glutamine, 10 mM HEPES) and Geneticin (500 μg/ml) at 37 °C under 5% CO2.

**Isolation of Murine Macrophages**—129sv mice expressing a wild-type allele at the Nramp1 locus, Nramp1G169/G169 (Nramp1-1/1) were originally obtained from Taconic Farms and subsequently maintained as a breeding colony according to regulations of the Canadian Council of Animal Care. Mice were sacrificed by cervical dislocation at the National Institutes of Health (NIH) formatation at the Nramp1 locus ( Nramp1-1/1) were produced by homologous recombination, as described previously (16). Resident peritoneal macrophages were obtained from 8- to 12-week-old wild-type (+/+) and knockout (-/-) mice by peritoneal lavage with 10 ml of sterile PBS, as described previously (21). The harvested cell suspension contained ~30% mature macrophages as determined by Wright staining. Cells were washed twice with PBS and resuspended at 10⁶/ml in medium RPMI 1640 containing 10% serum and 5 mM glutamine before plating on glass coverslips. Two hours later, nonadherent cells were removed by washing with fresh medium and macrophages were used 24 h later for Salmonella invasion and immuno-fluorescence assays.

**Bacterial Strains**—Two Salmonella strains were used for invasion studies. The SL1344 strain was used for pH measurements, and the 14028s strain expressing the pFPV1, a modified pBB322 plasmid encoding the green fluorescent protein (23), for fluorescence detection assays. InvA- cells, which lack InvA, an essential component of the Salmonella SPI-1 type III secretion apparatus needed for bacterial invasion, were used as noninvasive controls. All bacteria were grown in Luria-Bertani liquid medium supplemented with appropriate antibiotics. To label SL1344 cells with FITC, 300 μl of a bacterial suspension in exponential phase was sedimented, washed in PBS, and resuspended in 0.5 mg/ml FITC dissolved in 0.1 n NaHCO3, pH 8.8. This suspension was incubated at room temperature for 25 min in the dark, shaking. The bacteria were then washed in PBS and used immediately for invasion.

**Invasion Experiments**—To optimize invasion of RAW cells, Salmonella was grown as described by Francis et al. (24). Briefly, the bacteria were grown in Luria-Bertani with overnight shaking in Luria-Bertani at 37 °C. Salmonella were then subcultured at 1:25 for 3 h more at 37 °C to late log phase. After harvesting by centrifugation, the bacteria were washed with PBS, resuspended in Earle’s buffered saline solution (1.4 mM CaCl2, 0.8 mM MgSO4, 5.4 mM KCl, 160 mM NaCl, 5.5 mM glucose, 2 mM NaHCO3, 1 mM NaH2PO4, pH 7.7) and added (multiplicity of infection 1–100) to RAW cells grown to near confluence on glass coverslips. Invasion was allowed to proceed for 10 min at 37 °C and the coverslips were then washed four times to eliminate uninvatized bacteria.

**Measurement of pH in SCV**—After invasion by FITC-labeled Salmonella, RAW/Nramp1-1 and RAW/Nramp1-1 cells were transferred to buffer A (140 mM NaCl, 5 mM KCl, 10 mM glucose, 1 mM MgCl2, 1 mM CaCl2, 20 mM HEPES, pH 7.4) and incubated for 1 h at 37 °C. The coverslip was placed in a Leica DMIRB inverted microscope equipped for digital ratio imaging (25). Bacteria within the cells were identified by epifluorescence and could often be seen by differential interference contrast microscopy to be located within large vacuoles, particularly at early times. Intravacular pH was calculated from the ratio of fluorescence using 490 nm and 440 nm excitation, as described (25). Calibration was performed in situ by incubating the cells with nigericin and monensin (each at 5 μg/ml) in media of defined pH buffered in the 7.5 to 4.5 range.

**Immuno-fluorescence**—Immediately after invasion, cells infected with GFP-expressing Salmonella were incubated in RPMI buffer at 37 °C to allow for SCV maturation. After 30–90 min, maturation was terminated by addition of ice-cold PBS. To detect extracellular bacteria, the cells were incubated in the cold with anti-Salmonella LPS antibodies (1:200 dilution) for 15 min, washed and exposed to secondary antibodies labeled with Alexa Fluor 350 (1:200) and for an additional 15 min. After washing, the cells were fixed in 4% paraformaldehyde for 1 h, then permeabilized and blocked overnight in 1% donkey serum, 2% albumin plus 0.1% Triton X-100. To label late endosomes-lysosomes, cells were incubated with anti-LAMP1 (1:4) or anti-M6PR (1:800) in PBS plus 0.1% Triton X-100 for 1 h followed by Cy3-conjugated secondary antibodies for 60 min. Monoclonal anti-c-Myc antibodies were used to localize the epitope-tagged Nramp1 in transfected cells. In primary cells, Nramp1 was detected by incubation with the affinity-purified anti-Nramp1 antiserum (1:150) for 4 h, followed by a 1-h incubation with Cy3-conjugated goat anti-rabbit antibody (1:1500).

**Uptake of Rhodamine-dextran**—Two types of experiments were performed in cells labeled with a blocking buffer (1% albumin, 2% fetal bovine serum in PBS). To detect extracellular bacteria, the cells were incubated in the cold with anti-Salmonella LPS antibodies (1:200 dilution) for 15 min, washed and exposed to secondary antibodies labeled with Alexa Fluor 350 (1:200) for an additional 15 min. After washing, the cells were fixed in 4% paraformaldehyde for 1 h, then permeabilized and blocked overnight in 1% donkey serum, 2% albumin plus 0.1% Triton X-100. To label late endosomes-lysosomes, cells were incubated with anti-LAMP1 (1:4) or anti-M6PR (1:800) in PBS plus 0.1% Triton X-100 for 1 h followed by Cy3-conjugated secondary antibodies for 60 min. Monoclonal anti-c-Myc antibodies were used to localize the epitope-tagged Nramp1 in transfected cells. In primary cells, Nramp1 was detected by incubation with the affinity-purified anti-Nramp1 antiserum (1:150) for 4 h, followed by a 1-h incubation with Cy3-conjugated goat anti-rabbit antibody (1:1500).

**RESULTS**

Expression of Nramp1 in RAW Cells and Primary Macrophages—Two experimental systems were used to define the effect of Nramp1 on the maturation of the SCV: wild-type and Nramp1-transfected RAW264.7 cells and peritoneal macrophages isolated from 129sv mice expressing a wild-type allele at the Nramp1 locus (Nramp1G169/G169, Nramp1-1/1), or from mice bearing a null mutation at the Nramp1 locus (Nramp1-1/1), produced by homologous recombination as described previously (16). The expression of Nramp1 in these cells is illustrated in Fig. 1. Briefly, neither the wild-type RAW cells nor the macrophages from knockout mice have detectable levels of Nramp1, as anticipated. In contrast, Nramp1 was readily detectable in the wild-type peritoneal macrophages, and very similar levels

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were found to be expressed in the RAW cells stably transfected with Nramp1.

Effect of Nramp1 on the pH of SCVs—To measure intravacuolar pH, Salmonella were labeled with FITC. Because of the covalent nature of the reaction, it was important to ensure that the invasive properties of the bacteria remained unaltered. Although high concentrations of FITC were found to depress the invasiveness of Salmonella, we empirically found conditions that yielded sufficiently bright fluorescent signals (Fig. 2D) with no discernible effect on invasion. This was determined microscopically, differentiating intracellular from adherent extracellular bacteria using anti-LPS antibodies before and after permeabilization of the RAW/Nramp1− macrophages, as described by others (26, 27). As illustrated in Fig. 2, under the conditions used, FITC-labeled and unlabeled bacteria entered macrophages at comparable rates. That invasion, rather than phagocytosis, mediated the entry of the bacteria into macrophages is shown in Fig. 3. Although the invasive strain SL1344 gained access to the cell’s interior, the noninvasive invA− mutant remained extracellular and was removed almost entirely by our routine washing procedure.

The spectral properties of the FITC-labeled Salmonella are illustrated in Fig. 3A, where the ratio of fluorescence with excitation at 490 and 440 nm is plotted versus pH. The fluorescence ratio was used to minimize the artifactual effects of photobleaching and of changes in the focal plane. As predicted, a sigmoidal relationship between fluorescence ratio and pH was obtained, with a midpoint in the 6.0–6.5 range. This is consistent with the reported pK of fluorescein derivatives and renders the probe suitable for measurements of pH in the acidic environment expected to prevail in the SCV (14, 28).

When exposed for 10 min to FITC-labeled SL1344 Salmonella, RAW/Nramp1− and RAW/Nramp1+ cells were invaded to a comparable extent (an average of 10 versus 11 bacteria/cell). The results of four determinations of intravacuolar pH in the two cell types are summarized in Fig. 3B. In both instances, the intravacuolar pH approached the extracellular pH shortly after invasion, but a gradual acidification developed over the next 60 min, reaching a pH between 6.0 and 6.3. Loss of the fluorescent dye by photobleaching and/or metabolic events occurring within the vacuole precluded accurate measurements of pH beyond 60 min. Importantly, within the period monitored, there was no significant difference in pH between the SCVs of RAW/Nramp1− and RAW/Nramp1+ cells (Fig. 3B). Similar results were obtained using two separate clones of Nramp1-transfected RAW cells. We concluded that Nramp1 does not alter the pattern of SCV acidification, at least during the first hour following invasion.

Subcellular Localization of Nramp1—In view of the similarity of the pH recorded in the SCVs of RAW/Nramp1− and RAW/Nramp1+ cells, it was important to ascertain that the Nramp1 protein was in fact reaching the Salmonella vacuole. As reported previously (18), we found an extensive co-localization of Nramp1 with LAMP1 (Fig. 4, A and B), a marker or late endosomes and lysosomes (29). Because LAMP1-containing compartments are well documented to merge with the SCV, we anticipated that Nramp1 would similarly reach the vacuole. This prediction was verified in Fig. 4 (C and D), where intracellular Salmonella can be seen to be encapsulated in Nramp1-containing vacuoles. Between 60 and 90 min after invasion, up to 90% of intracellular Salmonella were found to colocalize with LAMP1 in RAW/Nramp1+ cells. Therefore, the similarity of the vacuolar pH in RAW/Nramp1− and RAW/Nramp1+ cells cannot be attributed to the failure of Nramp1 to reach the SCV under the conditions used.

Colocalization of Salmonella with Late-endosomal and Lysosomal Markers—The survival of intracellular pathogens, including Salmonella, has been associated with their ability to avoid the microbicidal environment of lysosomes. The enhanced capacity of RAW/Nramp1+ cells to control infection could therefore result from alterations in the maturation pattern of the microbial vacuole. This notion was tested by comparing the rate and extent of acquisition of several organelar markers by SCVs in RAW/Nramp1− and RAW/Nramp1+ cells.
Nramp1 Modifies the Salmonella Fusion with Endomembranes

To facilitate detection of the bacteria, we used Salmonella that stably express enhanced GFP. Fig. 5 (A–D) shows that LAMP1 is readily detectable on the membrane lining the SCVs in both RAW/Nramp1 and RAW/Nramp1<sup>−</sup> cells. Extensive quantitative analysis (counting 100 internalized bacteria/experiment, four experiments of each type) indicated that virtually all the SCVs contained LAMP1 at both 60 and 90 min after infection (Fig. 5E). There were no obvious differences in the staining intensity of RAW/Nramp1<sup>−</sup> and RAW/Nramp1<sup>+</sup> cells.

Because it is found both in late endosomes and in lysosomes (30, 31), the precise source of the LAMP1 that reaches the pre-lysosomal compartment of the endocytic pathway, yet is not present in mature lysosomes (32–34). Although LAMP1 is found in the SCVs, the M6PR was reported to be largely excluded from vacuoles containing live Salmonella (12, 13, 27).

**FIG. 3.** Measurement of the pH of SCV in RAW/Nramp1<sup>−</sup> and RAW/Nramp1<sup>+</sup> cells. A, calibration of fluorescence ratio versus pH. The fluorescence emission at 530 nm of live Salmonella labeled with FITC was measured with excitation at both 440 and 490 nm, at varying pH. Ordinate, ratio of fluorescence at 490/440 nm. B, RAW/Nramp1 (open circles) or RAW/Nramp1<sup>−</sup> (solid circles) were exposed to FITC-labeled Salmonella (SL1344) for 10 min at 37 °C. The pH of the resulting SCVs was monitored for the next 60 min by ratio microfluorimetry, as described under “Experimental Procedures.” Data are means ± S.E. of 40 individual determinations. C, fluorescence of FITC-labeled Salmonella of the invasive strain SL1344 associated with RAW cells following 10-min incubation. D, corresponding DIC image. E, fluorescence of FITC-labeled Salmonella of the noninvasive strain InvA<sup>−</sup> associated with RAW/Nramp1<sup>−</sup> cells following a 10-min incubation. F, corresponding DIC image. Arrows indicate the location of some of the bacteria.

Although small, the differences were statistically significant (p < 0.05).

**Distribution of the Mannose 6-Phosphate Receptor (M6PR)—** The M6PR traffics between the trans-Golgi network and the pre-lysosomal compartment of the endocytic pathway, yet is not present in mature lysosomes (32–34). Although LAMP1 is found in the SCVs, the M6PR was reported to be largely excluded from vacuoles containing live Salmonella (12, 13, 27).

We therefore tested the effect of Nramp1 on the distribution of M6PR. As reported for nonmyeloid cells, M6PR is rarely found in SCVs within cells devoid of Nramp1 (Fig. 7, A and B). After 60 min, only 7% of the vacuoles contained M6PR and this number increased only marginally after 90 min, to 13% (Fig. 7E). Care was taken to count only intracellular bacteria and not those adherent to the cell exterior. The location of the bacteria was routinely ascertained using antibodies before and after permeabilization, as above.

In contrast to Nramp1-deficient cells, a much larger fraction of SCVs were M6PR positive in Nramp1-expressing cells at both 60 and 90 min (Fig. 7, C and E). This is unlikely to result from clonal variation, because similar results were obtained using two independently isolated clones (c-Myc/Nramp1-transfectants behaved comparably (Fig. 8, A and B)). As also shown in Fig. 6, the marker gained access to the SCVs after 60 min. Of note, fusion of SCVs with lysosomes was observed in both RAW/Nramp1 and RAW/Nramp1<sup>−</sup> cells and the extent of fusion was only modestly higher in the RAW/Nramp1<sup>−</sup> cells (89.5 ± 0.5% and 89 ± 4.5% of positive vacuoles at 60 and 90 min, respectively; n = 3 experiments, each with 100 bacteria counted) than in RAW/Nramp1<sup>−</sup> cells (79.5 ± 0.5% and 78.6 ± 5.8%) (Fig. 6D).

Although small, the differences were statistically significant (p < 0.05).

**Effect of Nramp1 Expression on Access of Fluid Phase Markers to Preformed SCVs—** At least in HeLa cells, some time after formation, the SCVs appear to segregate themselves from the endocytic pathway of the host cell. Once generated, the SCV cannot be readily accessed by fluid-phase markers added subsequently, suggesting a limited interaction with endocytic structures formed de novo (12). We tested the effect of Nramp1 on this segregation process. Salmonella were allowed to invade RAW/Nramp1 and RAW/Nramp1<sup>−</sup> cells, and 90 min were allowed for maturation of the vacuole. At this time, rhodamine dextran was added extracellularly and its ability to reach the SCV was monitored after an additional 30 and 60 min. As in the case of HeLa cells, only a small fraction of the SCVs was accessible in cells lacking Nramp1 (Fig. 8, A–C and G). After 30 min, only 20 ± 2% of the SCVs were stained, and this number increased little after 60 min. In contrast, upward of 70% of the vacuoles were accessed by the fluid phase marker in Nramp1 expressing cells (Fig. 8, D–G). As before, clonal variation is unlikely to explain this difference, because the two Nramp1 transfectants behaved comparably (Fig. 8G). It therefore appears that Nramp1 counteracts the ability of the SCV to become segregated from the endocytic pathway.
Effects of Nramp1 on SCV Maturation in Primary Murine Macrophages—The in vitro experiments using transfected RAW cells suggest that Nramp1 can alter the fate of the SCV, which may contribute to its effect on bacterial resistance. It was important, however, to ascertain that the observed effects were not restricted to cultured cells heterologously transfected with Nramp1. To this end, we compared the behavior of resident peritoneal macrophages obtained from Nramp1-expressing (+/+ ) and knockout (−/− ) mice. The presence and absence, respectively, of Nramp1 in the peritoneal cells was verified by immunostaining (not shown). Next, the acquisition of M6PR and the access of dextran to preformed SCVs was studied in infected primary cells as described above for RAW cells. A summary of the results obtained is presented in Fig. 9. The differences noted earlier in transfected RAW cells were not only reproduced, but somewhat greater in the peritoneal macrophages. In four experiments, the acquisition of M6PR by the vacuoles was nearly 4-fold higher in Nramp1−/− cells than in their Nramp1+/− counterparts (Fig. 9). More strikingly, access of labeled dextran to the preformed SCVs was 7–8-fold greater in Nramp1−/− than in Nramp1+/− cells. These observations are consistent with those described in immortalized cultured macrophages, and they jointly indicate that Nramp1 effectively modulates the vesicular traffic that underlies SCV maturation.

**DISCUSSION**

The pathogenesis of *Salmonella* infections is related to the ability of these bacteria to survive inside macrophages. To survive, *Salmonella* are thought to counteract degradation by the host cells by two mechanisms: delayed acidification of the SCV and its incomplete fusion with lysosomes. Although this concept has reached consensus in general terms, there is disagreement as to the extent of the acidification and the stage at which maturation is arrested. These topics are discussed below in the context of our results.

**Acidification of the SCV**—Early studies indicated that SCVs do not reach the extremely low pH encountered in mature lysosomes, prompting the notion that bacterial survival is dependent on its ability to actively attenuate vacuole acidification (14). However, subsequent studies failed to confirm this active modulation of vacuolar pH by *Salmonella*. Rathman et al. (28) found no significant difference in the acidification of vacuoles containing live or dead bacteria. Although these apparent inconsistencies could be attributed to differences in the experimental model used, it is clear that the absolute value and functional role of the pH within the SCV require additional study.

Under our experimental conditions, the SCV underwent a rapid, readily detectable acidification. During the course of 1 h, the pH dropped from the extracellular level of 7.4 to 6.2. The development of this acidification correlated with the acquisition of LAMP1 and pre-internalized fluid phase markers, indicating the fusion of the SCV with late endosomal/lysosomal compartments. To the extent that vacuolar ATPases coexist with LAMP1 in these compartments, the development of acidification is not unexpected. During the period of observation, the vacuolar pH did not reach the low pH (4.5–5.5) believed to exist in the lumen of primary lysosomes. Because reliable pH measurements could not be obtained beyond 1 h, it remains unclear whether a greater acidification would be reached at longer times.

**Fusion with Endomembrane Compartments**—The stage at which SCV maturation becomes arrested is also the subject of...
controversy. Although most authors find that the vacuoles reach at least the late endosomal stage, characterized by recruitment of Rab7 (35), others believe that the arrest occurs earlier, with accumulation of Rab5, a marker of sorting endosomes (36).

In macrophages we found that SCVs fuse with LAMP1-containing vesicles and with late components of the endocytic pathway preloaded with fluid phase markers. On the other hand, the SCV was largely inaccessible to M6PR or to fluid phase markers added some time after invasion. These results are consistent with earlier studies (12, 13). Because both LAMP1 and M6PR are found in late endosomes/pre-lysosomes, these findings are seemingly incompatible. However, they can be explained by assuming that the SCV can undergo fusion with most or all elements of the endocytic pathway, but only for a short period following invasion. Segregation of the vacuoles at later stages into a compartment that no longer fuses with endosomes or lysosomes would readily explain the failure of extracellular dextran to reach preformed SCVs. The much reduced M6PR content, despite the fusion with late endosomes/lysosomes, can be explained if the rate of delivery of this receptor from the trans-Golgi network to the late endosomes is comparatively slow. Thus, only a small, often undetectable amount of M6PR resident in the late endosomes would be delivered to the SCV during the finite period when endosomal fusion takes place. The restricted period of fusion would also limit the extent of fusion with lysosomal components, which is likely low but not negligible.

**Effect of Nramp1 on SCV Maturation**—During the period analyzed, the expression of Nramp1 in the infected macrophages had little effect on the vacuolar pH. The occurrence of substantial acidification in the SCVs is consistent with their acquisition of LAMP1, which co-exists with v-ATPases in late compartments of the endocytic pathway. We were unable to accurately measure vacuolar pH after 60 min of invasion and cannot rule out that difference in SCV acidification would develop thereafter in the presence and absence of Nramp1. Nevertheless, it is apparent that even in Nramp1-expressing transformed with a plasmid expressing GFP driven by the PhoP/Q promoter indicate that induction also occurs in the vacuole of Nramp1-expressing macrophages (data not shown).

Despite the activation of PhoP/Q, the maturation of the SCV...
is clearly modified by the activity of Nramp1. The SCVs formed in Nramp1-deficient cells fail to acquire M6PR and were not accessible to fluid phase markers once generated, suggesting

that the viable S. typhimurium had diverted the normal degradative pathway of the phagosome, which may subsequently avoid the killing by the host cells. Expression of Nramp1 clearly corrected the unusual trafficking pattern of SCVs, in that SCVs became positive for M6PR and were accessible to extracellular dextran. It is interesting to note that in Nramp1-deficient RAW cells phagosomes containing latex beads or heat-killed Salmonella also acquired M6PR and were accessible to extracellular dextran (13). The function of M6PR is to deliver a subset of lysosomal enzymes from the trans-Golgi network to the pre-lysosomal compartment. By facilitating access to M6PR and its cargo of lysosomal enzymes to the vacuole, Nramp1 may facilitate bacterial killing. This mechanism may explain, at least partially, the Nramp1-mediated host resistance to Salmonella infections.

The precise mechanism whereby Nramp1 alters vacuolar maturation is obscure, but recent observations suggest reasonable alternatives. Some insight into the possible mode of action of Nramp1 was gleaned from studies of Nramp2, a closely related isoform. Electrophysiological and isotopic determinations revealed that Nramp2 transports divalent cations such as Fe²⁺, Zn²⁺, and Mn²⁺ across the plasma membrane in a pH-dependent manner (38). More recently, spectroscopic studies of Mn²⁺ transport across the phagosomes of Nramp1-mediated host resistance to Salmonella infections.

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vacuolar lumen of metals that may be essential for bacterial survival. It is noteworthy that Nramp-related transporters have been found to be expressed by a variety of bacteria, including Salmonella (39–41), which in all likelihood use these molecules to concentrate Fe$^{2+}$, Mn$^{2+}$, and/or other metals in their cytosol. If accumulation of such metals by the bacteria is required for the expression or function of their pathogenic gene products, including those that divert the SCV from the endocytic pathway, the antagonistic effect of Nramp1 can be readily rationalized.

In summary, we found that expression of Nramp1 confers resistance to Salmonella, at least in part, by altering the pattern of maturation of the SCV. Nramp1 likely exerts this effect by actively removing from the vacuolar lumen metal ions that are essential for pathogen survival and proliferation. The precise identity of the ions, as well as the bacterial gene products that are counteracted by Nramp1, remain obscure and require additional study.

REFERENCES

1. Pang, T., Bhutta, Z. A., Finlay, B. B., and Altwegg, M. (1995) Trends Microbiol. 3, 253
2. Collazo, C. M., Zierler, M. K., and Galan, J. E. (1995) Mol. Microbiol. 15, 25–38
3. Oehman, H., Soncini, F. C., Solomon, F., and Groisman, E. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7800–7804
4. Shea, J. E., Hensel, M., Gleeson, C., and Holden, D. W. (1996) Mol. Microbiol. 30, 175–188
5. Hensel, M., Shea, J. E., Waterman, S. R., Mundy, R., Nikolaus, T., and Banks, G. (1998) Mol. Microbiol. 30, 163–174
6. Blanc-Potard, A. B., and Groisman, E. A. (1997) EMBO J. 16, 5376–5385
7. Pfeifer, C. G., Marcus, S. L., Steele-Mortimer, O., Knodler, L. A., and Finlay, B. B. (1999) Infect. Immun. 67, 2225–2232
8. Hom, K. H., and Miller, V. L. (1998) J. Bacteriol. 180, 1793–1802
9. Hardt, W. D., Chen, L. M., Shuebel, K. E., Bustelo, X. R., and Galan, J. E. (1998) Cell 93, 415–426
10. Pfeifer, C. G., Marcus, S. L., Steele-Mortimer, O., Knodler, L. A., and Finlay, B. B. (1999) Infect. Immun. 67, 5690–5698
11. Desjardins, M., Huber, L. A., Parton, R. G., and Griffiths, G. (1994) J. Cell Biol. 124, 677–688
12. Garcia del Portillo, F., and Finlay, B. B. (1995) J. Cell Biol. 129, 81–97
13. Rathman, M., Barker, L. P., and Falkow, S. (1997) Infect. Immun. 65, 1475–1485
14. Alpuche-Aranda, C. M., Swanson, J. A., Loomis, W. P., and Miller, S. I. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10079–10083
15. Vidal, S. M., Malo, D., Vogan, K., Skamene, E., and Gros, P. (1993) Cell 73, 469–485
16. Vidal, S. M., Tremblay, M. L., Govoni, G., Gauthier, S., Sebastiani, G., Malo, D., Skamene, E., Olivier, M., Jothy, S., and Gros, P. (1995) J. Exp. Med. 182, 555–566
17. Vidal, S. M., Gros, P., and Skamene, E. (1995) J. Leukocyte Biol. 58, 382–390
18. Gruenheid, S., Pinner, E., Desjardins, M., and Gros, P. (1997) J. Exp. Med. 185, 717–730
19. McFadden, G. I., Roach, T. I., A. A., Atkinson, P. G. P., Barton, C. H., Meleo, R. H., and Blackwell, J. M. (1990) J. Cell Biol. 111, 2855–2866
20. Govoni, G., and Gros, P. (1998) Inflamm. Res. 47, 277–284
21. Jabado, N., Jankowski, A., Dougapassard, S., Picard, V., Grinstein, S., and Gros, P. (2000) J. Exp. Med. 192, 1237–1248
22. Govoni, G., Cannone-Hergaux, F., Pfeifer, F., Marcus, S. L., Mills, S. D., Hackman, D. J., Grinstein, S., Malo, D., Finlay, B. B., and Gros, P. (1999) Infect. Immun. 67, 2225–2232
23. Valdivia, R. H., Hromocky, A. E., Monack, D., Ramakrishnan, L., and Falkow, S. (1996) Gene (Amst.) 175, 47–52
24. Francis, C. L., Starnbach, M. N., and Falkow, S. (1992) Mol. Microbiol. 6, 3077–3087
25. Demaurex, N., Romanek, R., Rotstein, O., and Grinstein, S. (1998) Cell Biology: A Laboratory Handbook, 2nd Ed, pp. 380–386, Academic Press, San Diego
26. Coppolino, M. G, Kong, C., Mohtashami, M., Schreiber, A. D., Brunnell, J. H., Finlay, B. B., Grinstein, S., and Trimble, W. S. (2001) J. Biol. Chem. 276, 4772–4780
27. Steele-Mortimer, O., Mesere, S., Gervel, J. P., Tob, B. H., and Finlay, B. B. (1999) Cell Microbiol. 1, 33–49
28. Rathman, M., Sjaastad, M., and Falkow, S. (1996) Infect. Immun. 64, 2765–2773
29. Kornfeld, S., and Mellman, I. (1996) Annu. Rev. Cell Biol. 5, 348–345
30. Green, S. A., Zimmer, K. P., Griffiths, G., and Mellman, I. (1987) J. Cell Biol. 105, 1227–1240
31. Braun, M., Waheed, A., and van Figura, K. (1989) EMBO J. 8, 3633–3640
32. Griffiths, G., Hoflack, B., Simons, K., Mellman, I., and Kornfeld, S. (1988) Cell 52, 329–341
33. Griffiths, G., Matteoni, R., Back, R., and Hoflack, B. (1990) J. Cell Sci. 95, 441–461
34. Rabinovitz, S., Hortsman, H., Gordon, S., and Griffiths, G. (1992) J. Cell Biol. 116, 95–112
35. Mesere, S., Steele-Mortimer, O., Finlay, B. B., and Gevel, J. P. (1999) EMBO J. 18, 4384–4403
36. Hashim, S., Muberek, K., Jais, A., Basu, S. K., and Mukhopadhay, A. (2000) Process Microbiol. 47, 482–486
37. Kehres, D. G., Zaharick, M. L., Finlay, B. B., and Maguire, M. E. (2000) Mol. Microbiol. 35, 1065–1078
38. Keles, D. G., Zaharick, M. L., Finlay, B. B., and Maguire, M. E. (2000) Mol. Microbiol. 36, 1085–1100
39. Malu, H., Roig, E., Cole, S. T., Helmann, J. D., and Gros, P. (2000) Mol. Microbiol. 35, 1065–1078
40. Agranoff, D., Monahan, I. M., Mangani, J. A., Butcher, P. D., and Krishna, S. (1999) J. Exp. Med. 190, 717–724
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