Regulation of Nramp1 mRNA stability by oxidants and protein kinase C in RAW264.7 macrophages expressing *Nramp1*^{Gly169}

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The murine *Nramp1* (natural-resistance-associated macrophage protein) locus confers innate resistance against intracellular macrophage pathogens. The gene encodes a transporter molecule, which is rapidly recruited to the phagosome. Nramp1 functions as an iron transporter by transporting iron into the phagosome. Within the phagosome iron mediates anti-microbial killing by hydroxyl radical formation through the iron-catalysed Fenton/Haber–Weiss reaction. In addition to its effects on the growth of intracellular pathogens, *Nramp1* exerts a wide range of pleiotropic effects in activated macrophages. One of these pleiotropic effects is the increased stability of a variety of mRNA species, including Nramp1 mRNA. In the present study, the stability of Nramp1 mRNA in *Mycobacterium avium* infected RAW264.7 macrophages expressing either the *Nramp1*^{Gly169} resistant allele or the *Nramp1*^{Asp169} susceptible allele was examined. Nramp1 mRNA stability was greater in *Nramp1*^{Gly169} macrophages than

in *Nramp1*^{Asp169} macrophages. The increase in Nramp1 mRNA stability in resistant macrophages was inhibited by antioxidants and protein kinase C (PKC) inhibitors, suggesting that Nramp1 mRNA stability is regulated by an oxidant-generated signalling pathway that requires PKC activity. This was corroborated by treating *Nramp1*^{Asp169} macrophages with menadione, which generates reactive oxygen species within cells. Menadione increased Nramp1 mRNA stability to the level observed in resistant macrophages; this increase was also inhibited by a PKC inhibitor. Further, PKC activity was found to be greater in *M. avium*-infected *Nramp1*^{Gly169} macrophages than in infected *Nramp1*^{Asp169} macrophages and inhibited by treatment with an antioxidant.

Key words: innate resistance, *Mycobacterium avium*, oxidative stress.

INTRODUCTION

The murine *Nramp1* (natural-resistance-associated macrophage protein) locus determines innate resistance to a number of intracellular pathogens including *Mycobacterium avium*, *M. bovis*, *M. lepraemurium*, *Leishmania donovani* and *Salmonella typhimurium* [1–6]. *Nramp1* is expressed in monocytes/macrophages and granulocytes, where it restricts the growth of intracellular pathogens [3,6]. The predicted amino acid sequence indicates that Nramp1 is an integral membrane protein with 10–12 transmembrane segments, typical of eukaryotic and prokaryotic transporter molecules [3,7]. Susceptibility to intracellular pathogens is associated with a non-conservative mutation involving a Gly^{resistant} to Asp^{susceptible} substitution at position 169 in the fourth membrane-spanning domain [3].

Nramp1 expression in macrophages is up-regulated by bacterial lipopolysaccharide (LPS), the cytokines interferon γ (IFN- γ) and granulocyte/macrophage colony-stimulating factor ('GM-CSF'), and inflammatory stimuli [6,8]. Biochemical studies showed that Nramp1 is a 90–110 kDa phosphoglycoprotein expressed in vesicles of the endocytic pathway [9–12]. On phagocytosis, Nramp1 is rapidly recruited to the phagosome and remains associated with the phagosome throughout phagolysosome maturation. The presence of Nramp1 within the phagosome membrane suggests that Nramp1 might alter the micro-environment of the phagosome to affect microbial killing [13].

Structural similarities between Nramp1 and prokaryotic and eukaryotic transporters suggest that Nramp1 is an ion transporter [3,7,14]. Nramp1 also has 64% sequence identity with a second

Nramp protein, Nramp2, which has been shown to be the iron uptake system within the intestine [14–17]. Ion transport studies in oocytes expressing Nramp2 also showed that Nramp2 transports a number of bivalent cations, including Fe²⁺, Mn²⁺, Zn²⁺ and Cu²⁺ [18]. Recently, a study in our laboratory showed that Nramp1 also functions as an iron transporter by transporting iron into the phagosome [19]. In these studies we showed that the import of iron *in vitro* into phagosomes isolated from RAW264.7 macrophages transfected with the resistant *Nramp1*^{Gly169} allele was more than twice the import of iron into phagosomes isolated from RAW264.7 macrophages transfected with the susceptible *Nramp1*^{Asp169} allele. In addition, phagosomes isolated from resistant cells that had been prelabelled with radiolabelled iron contained up to 4-fold more iron than phagosomes from prelabelled susceptible cells. Within the phagosome, iron can catalyse the formation of hydroxyl radicals from H₂O₂ via the Fenton/Haber–Weiss reaction. The role of iron and hydroxyl radicals in anti-microbial activity was also examined [20]. The addition of small amounts of iron to macrophages from *Nramp1* resistant mice increased the capacity of the macrophages to inhibit the growth of *M. avium*. In contrast, the addition of iron to macrophages from *Nramp1* susceptible mice increased the growth of the mycobacteria. Treatment of macrophages from resistant mice with inhibitors of hydroxyl radical generation prevented the enhancing effect of iron on the growth inhibitory capacity of the macrophages. We conclude from these studies that iron transport by Nramp1 into the phagosome mediates anti-microbial killing by hydroxyl radical formation through the iron-catalysed Fenton/Haber–Weiss reaction.

Abbreviations used: BHA, butylated hydroxyanisole; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; IFN- γ , interferon γ ; LPS, lipopolysaccharide; PDTTC, pyrrolidinedithiocarbamate; PKC, protein kinase C; UTR, untranslated region.

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In addition to its effects on the growth of intracellular pathogens, *Nramp1* exerts a wide range of pleiotropic effects in IFN- γ /LPS-activated macrophages, including increased expression of the chemokine KC, interleukin-1 α , tumour necrosis factor α and cell-surface MHC class II [21–24]. Also increased in *Nramp1* resistant macrophages is the respiratory burst, the release of nitrates, L-arginine fluxes and protein kinase C (PKC) activity [24–26]. Previous studies from this laboratory have shown that a variety of mRNA species, including *Nramp1* mRNA, are more stable in IFN- γ -activated *Nramp1* resistant macrophages than in *Nramp1* susceptible macrophages [8,27]. This suggests that some of the pleiotropic effects might be due to increased expression resulting from increased mRNA stability. In the present study we examined the stability of *Nramp1* mRNA in macrophages that have phagocytosed the pathogen *M. avium*. We found that *Nramp1* mRNA stability was higher in *M. avium*-infected RAW264.7 cells expressing the *Nramp1* resistant allele than in RAW264.7 cells expressing the *Nramp1* susceptible allele. We also show that the increased *Nramp1* mRNA stability in resistant macrophages results from an oxidant-generated signalling pathway that requires PKC activity.

EXPERIMENTAL

Reagents

M. avium (35713) was obtained from the American Type Culture Collection (Manassas, VA, U.S.A.) and cultured as described previously [8]. Actinomycin D, menadione, butylated hydroxy-anisole (BHA), desferrioxamine and pyrrolidinedithiocarbamate (PDTC) were purchased from Sigma (St Louis, MO, U.S.A.). Bisindolylmaleimide I and Gö6976 were obtained from Calbiochem (La Jolla, CA, U.S.A.). The probe for *Nramp1* was a 576 bp *Pst*I fragment isolated from an *Nramp1* cDNA clone that we cloned from a macrophage cDNA library. The glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) probe was isolated from a cDNA subtraction library of IFN- γ -stimulated macrophages. [³²P]dCTP (specific radioactivity 3000 Ci/mmol) was purchased from Amersham (Arlington Heights, IL, U.S.A.).

Macrophage cultures

The RAW264.7 mouse macrophage cell lines stably transfected with *Nramp1*^{Gly 169} (7.5R) or *Nramp1*^{Asp 169} (10.S) were kindly provided by Jenefer Blackwell (Cambridge University, Cambridge, U.K.). These cell lines were created [25] by cloning *Nramp1* cDNA species in the pBabe vector, which contains a puromycin resistance marker under the control of a simian virus 40 early promoter, and introducing them by electroporation into the RAW264.7 macrophage cell line. These cells express *Nramp1* transgenes under the control of a viral long terminal repeat, 1.1TR, and were established as stable cell lines by puromycin resistance. Functional analysis of these cell lines has shown that the cell line containing the resistant *Nramp1*^{Gly 169} allele expresses the *Nramp1* phenotype on macrophage activation with IFN- γ /LPS, as exhibited by enhanced respiratory burst, nitrite release and MHC class II expression compared with the cell line expressing the susceptible *Nramp1*^{Asp 169} allele [25].

The cells were maintained in Dulbecco's minimum essential medium ('DMEM'; Gibco BRL, Gaithersburg, MD, U.S.A.) supplemented with 15% (v/v) fetal bovine serum (less than 3 ng/ml LPS; HyClone, Logan, UT, U.S.A.), 5 mM sodium pyruvate and penicillin/streptomycin. The cells were tested periodically for plasmid retention by growth in puromycin and were not used in culture for longer than 3 months.

Analysis of mRNA stability in *M. avium*-infected macrophages

The RAW264.7 cell lines were cultured in six-well tissue-culture plates for 4–6 h at 5×10^6 cells per well. The cells were then infected for 16 h with *M. avium* at a bacteria-to-macrophage ratio 10:1. To determine mRNA half-lives, actinomycin D (2 μ g/ml) was added and RNA was isolated by the method of Chomczynski and Sacchi [28] at 0, 3, 6, 9 and 12 h. Ideally, decay curves should have time points that extend beyond the half-life. However, durations beyond 12 h were not possible with RAW264.7 cells, because these cells are susceptible to actinomycin D toxicity and begin to die after that time. The effect of antioxidants and PKC inhibitors on mRNA stability was examined by the addition of inhibitors 30 min before treatment with actinomycin D.

Northern blot hybridization

RNA (15 μ g per lane) was separated on formaldehyde/1% (w/v) agarose gels and transferred by capillary blotting to Hybond-N⁺ membranes (Amersham). Northern blot hybridization was performed as described previously [29]. Gel-purified *Nramp1* and *G3PDH* cDNA probes were labelled with [³²P]dCTP by the random primer method (High Prime, Boehringer Mannheim, Indianapolis, IN, U.S.A.). Membranes were washed twice at 42 °C in $2 \times$ SSC/0.50% SDS for 20 min and once in $0.50 \times$ SSC/0.50% SDS for 40 min. Membranes were exposed for 2–3 days to Kodak BioMax MR film with intensifying screens. Autoradiographs were semiquantified by scanning with an HP scanner with the use of SigmaScan Pro 4 (SPSS, Chicago, IL, U.S.A.). *Nramp1*-specific signals were normalized to the signal obtained with the probe for *G3PDH* to account for differences in loading and a decline in mRNA that might have been due to actinomycin D toxicity. The mRNA half-lives for each individual experiment were calculated by a linear regression analysis of semilogarithmic plots of the percentage of *Nramp1* mRNA remaining against time after the addition of actinomycin D. The regression coefficient, r^2 , was consistently 0.90 or greater, indicating a linear relationship between the percentage of *Nramp1* mRNA decay and time.

Measurement of PKC activity

The PKC activity was determined in the protein particulate fraction after chromatography on DE52 cellulose columns. In brief, cells were harvested and homogenized in 20 mM Tris/HCl (pH 7.5)/0.50 mM EDTA/0.50 mM EGTA/10 mM 2-mercaptoethanol/1 μ g/ml leupeptin/0.70 μ g/ml pepstatin/1 μ g/ml aprotinin/1 mM PMSF. After ultracentrifugation at 100000 g and 4 °C, the membrane pellet was resuspended in the above buffer containing 1% (v/v) Nonidet P40. After 15 min on ice, the suspension was centrifuged at 19000 g and 4 °C. The supernatants were applied to 0.50 ml DE52 columns equilibrated in 20 mM Tris/HCl (pH 7.5)/0.50 mM EDTA/0.50 mM EGTA/10 mM 2-mercaptoethanol. After the columns had been washed with buffer, fractions containing PKC were eluted with buffer containing 0.3 M NaCl. Protein concentration was determined by the Bradford method (Bio-Rad, Richmond, CA, U.S.A.). PKC activity was determined in triplicate by measuring the transfer of radioactive phosphate from [γ -³²P]ATP to a specific peptide substrate (QKRPSQRSKYL; single-letter amino acid codes) in accordance with the manufacturer's instructions (Upstate Biotechnology, Lake Placid, NY, U.S.A.). After 10 min at 30 °C, samples were spotted on P81 phosphocellulose discs

and washed in 0.75 % (v/v) phosphoric acid; radioactivity was then counted by scintillation. Net specific PKC radioactivity was calculated by subtracting the ^{32}P incorporated in the presence of PKC inhibitor peptide, which corresponds to the inhibitory pseudosubstrate sequence (amino acid residues 19–31) of PKC.

RESULTS

Differential Nramp1 mRNA stability in macrophage cell lines expressing *Nramp1*^{Gly169} or *Nramp1*^{Asp169}

To determine whether Nramp1 mRNA stability differs between RAW264.7 cell lines expressing either resistant *Nramp1*^{Gly169} or susceptible *Nramp1*^{Asp169}, the cell lines were infected with *M. avium* for 16 h to up-regulate the expression of Nramp1 mRNA (Figure 1). Expression of Nramp1 mRNA was increased by *M. avium* infection in both *Nramp1*^{Gly169} and *Nramp1*^{Asp169} cells. However, the level of Nramp1 mRNA was higher in the cell line expressing *Nramp1*^{Gly169}. A Western blot analysis of Nramp1 protein expression also showed higher levels of Nramp1 protein after *M. avium* infection of *Nramp1*^{Gly169} macrophages than in *Nramp1*^{Asp169} macrophages (results not shown). After the addition of actinomycin D, RNA was isolated at various times and Nramp1 mRNA decay was determined by Northern Blot

Figure 1 *M. avium* infection induces higher levels of Nramp1 mRNA in *Nramp1*^{Gly169} macrophages than in *Nramp1*^{Asp169} macrophages

(A) RAW264.7 macrophages expressing either *Nramp1*^{Gly169} or *Nramp1*^{Asp169} were infected with *M. avium* (bacteria-to-macrophage ratio 10:1) for 16 h. RNA was isolated and Northern blots were hybridized with cDNA probes for *Nramp1* and *G3PDH*. Results are representative of three experiments. (B) Densitometry of mRNA levels shown in (A): open columns, *Nramp1*^{Gly169}; hatched columns, *Nramp1*^{Asp169}. Values are the relative *Nramp1* levels obtained by dividing the *Nramp1* signal by the *G3PDH* signal.

Figure 2 Nramp1 mRNA is more stable in *Nramp1*^{Gly169} macrophages than in *Nramp1*^{Asp169} macrophages

RAW264.7 macrophages expressing either *Nramp1*^{Gly169} or *Nramp1*^{Asp169} were infected for 16 h with *M. avium* (bacteria-to-macrophage ratio 10:1). After the addition of actinomycin D (2 $\mu\text{g}/\text{ml}$), RNA was isolated at 0, 3, 6, 9 and 12 h. (A) Representative Northern blots hybridized with *Nramp1* and *G3PDH* probes. Numbers under the blots are Nramp1 mRNA levels determined by a densitometric analysis of the *Nramp1* and *G3PDH* blots. The *Nramp1* signal was normalized to the *G3PDH* signal and the percentage of Nramp1 RNA present relative to RNA at zero time was determined. (B) Decay curves of Nramp1 mRNA, showing the percentage of mRNA remaining after treatment with actinomycin D, were calculated from a densitometric analysis of the *Nramp1* and *G3PDH* Northern blots. Symbols: ●, *Nramp1*^{Gly169}; ○, *Nramp1*^{Asp169}. The results are means $\pm$ S.E.M. for the pooled data from 11 separate experiments.

hybridization to *Nramp1* and *G3PDH* cDNA probes (Figure 2A). Nramp1 mRNA was more stable in the cell line expressing resistant *Nramp1*^{Gly169} than in the *Nramp1*^{Asp169} cell line (Figures 2A and 2B). Nramp1 mRNA half-lives were determined by a linear regression of semilogarithmic plots of Nramp1 mRNA decay. There was a 2-fold difference in Nramp1 mRNA half-life between resistant *Nramp1*^{Gly169} macrophages and susceptible *Nramp1*^{Asp169} macrophages (25.1 ± 4.6 h compared with 11.6 ± 1.8 h; results given as means $\pm$ S.D., $P < 0.0001$). This difference in Nramp1 mRNA half-life is identical to that previously reported in IFN- γ -stimulated macrophages from *B6* resistant and susceptible mice [27].

Nramp1 mRNA stability is inhibited by antioxidants and iron chelation in resistant *Nramp1*^{Gly169} macrophages

Previous studies have shown that *Nramp1* resistant macrophages during a respiratory burst produce substantially more reactive oxygen species than *Nramp1* susceptible macrophages [19,25].

Figure 3 *Nrampl* mRNA stability is inhibited by antioxidants in *Nrampl*^{Gly169} macrophages

Nrampl^{Gly169} and *Nrampl*^{Asp169} macrophages were infected with *M. avium* for 16 h. Antioxidants PDTC (1 mM) and BHA (250 μ M) were added 30 min before the addition of actinomycin D. RNA was isolated at 0, 3, 6, 9 and 12 h after the addition of actinomycin D. Northern blots (**A**, **B**) were hybridized with *Nrampl* and *G3PDH* cDNA: (**A**) *Nrampl*^{Gly169}; (**B**) *Nrampl*^{Asp169}. Numbers under *Nrampl* blots are *Nrampl* mRNA levels relative to zero time determined by a densitometric analysis of the *Nrampl* and *G3PDH* blots and calculated as described in the legend to Figure 1. Decay curves of *Nrampl* mRNA from *Nrampl*^{Gly169} macrophages (**C**) and *Nrampl*^{Asp169} macrophages (**D**) were determined from a densitometric analysis of the Northern blots hybridized with *Nrampl* and *G3PDH* probes. Symbols: ●, control; ○, PDTC; ▼, BHA. Results are means $\pm$ S.E.M. for four separate experiments. (**E**) Half-lives of *Nrampl* mRNA determined from the decay curves. Open columns, control; hatched columns, PDTC; grey columns, BHA. Results are means $\pm$ S.D. for the four experiments. BHA and PDTC significantly decreased the half-life of *Nrampl* mRNA in the *Nrampl*^{Gly169} macrophages ($P < 0.005$, Student's *t* test).

Because reactive oxygen species have been implicated in intracellular signalling pathways, we hypothesized that oxidants might trigger a signalling pathway leading to higher *Nrampl* mRNA stability in resistant *Nrampl*^{Gly169} macrophages. To test this hypothesis, resistant and susceptible macrophages were treated with the antioxidants PDTC and BHA for 30 min before the addition of actinomycin D. Both antioxidants increased the decay of *Nrampl* mRNA in the resistant *Nrampl*^{Gly169} macrophages (Figures 3A and 3C) but had no effect on the decay of *Nrampl* mRNA in the susceptible *Nrampl*^{Asp169} macrophages (Figures 3B and 3D). The antioxidants decreased *Nrampl*

mRNA half-lives in *Nrampl*^{Gly169} macrophages to the level observed in *Nrampl*^{Asp169} macrophages (Figure 3E).

The effect of iron chelation was examined by treating macrophages with desferrioxamine for 30 min before the addition of actinomycin D. Iron chelation also increased the decay of *Nrampl* mRNA in the resistant *Nrampl*^{Gly169} macrophages (Figures 4A and 4C) but had no effect on the decay of *Nrampl* mRNA in the susceptible *Nrampl*^{Asp169} macrophages (Figures 4B and 4C). The *Nrampl* mRNA half-life was also decreased by iron chelation in *Nrampl*^{Gly169} macrophages to the level observed in *Nrampl*^{Asp169} macrophages (Figure 4D).

Figure 4 Inhibition of Nramp1 mRNA stability in *Nramp1*^{Gly169} macrophages by iron chelation

Nramp1^{Gly169} and *Nramp1*^{Asp169} macrophages were infected with *M. avium* for 16 h. The iron chelator desferrioxamine (Des; 50 μ M) was added 30 min before the addition of actinomycin D. RNA was isolated at 0, 3, 6, 9 and 12 h after the addition of actinomycin D. Northern blots (A, B) were hybridized with *Nramp1* and *G3PDH* cDNA probes: (A) *Nramp1*^{Gly169}; (B) *Nramp1*^{Asp169}. Numbers under *Nramp1* blots are Nramp1 mRNA levels relative to zero time determined by a densitometric analysis of the *Nramp1* and *G3PDH* blots and calculated as described in the legend to Figure 1. (C) Nramp1 mRNA decay curves derived from a densitometric analysis of the Northern blot hybridizations. Symbols: ●, *Nramp1*^{Gly169} control; ○, *Nramp1*^{Gly169} with desferrioxamine; ▼, *Nramp1*^{Asp169} control; ▽, *Nramp1*^{Asp169} with desferrioxamine. Results are means $\pm$ S.E.M. for four separate experiments. (D) Nramp1 mRNA half-lives derived from the decay curves. Open columns, control; hatched columns, desferrioxamine. Results are means $\pm$ S.D. for the four experiments. Desferrioxamine significantly inhibited Nramp1 mRNA half-lives in *Nramp1*^{Gly169} macrophages ($P < 0.005$, Student's *t* test).

Nramp1 mRNA stability in *Nramp1*^{Gly169} macrophages is dependent on PKC signalling

Previous studies from this laboratory and others have shown that *Nramp1* resistant macrophages activated by IFN- γ or *M. bovis* infection have significantly higher PKC activity than *Nramp1* susceptible macrophages [24,26]. To determine whether the stability of Nramp1 mRNA in resistant macrophage is dependent on PKC signalling, PKC inhibitors were added 30 min before the addition of actinomycin D. Two different PKC inhibitors were used. Bisindolylmaleimide I acts as a competitive inhibitor for the ATP-binding site of PKC and has a high selectivity for the α , β I, β II, γ , δ and ϵ isoforms of PKC [30]. Gö6976 inhibits the Ca²⁺-dependent α and β isoforms of PKC with no effect on the Ca²⁺-independent PKC isoforms [31]. Both PKC inhibitors increased Nramp1 mRNA decay in *Nramp1*^{Gly169} macrophages (Figures 5A and 5C) and had no effect on Nramp1 mRNA decay in *Nramp1*^{Asp169} macrophages (Figures 5B and 5D). The Nramp1 mRNA half-life in the resistant macrophages

was decreased by the PKC inhibitors to that in susceptible macrophages (Figure 5E).

Generation of reactive oxygen species in susceptible *Nramp1*^{Asp169} macrophages increases Nramp1 mRNA stability by a PKC-dependent pathway

The above experiments suggest that the differential Nramp1 mRNA stability in resistant and susceptible macrophages results from an oxidant-generated signalling pathway that involves Ca²⁺-dependent PKC. We next determined whether the generation of reactive oxygen species in *Nramp1*^{Asp169} macrophages increases Nramp1 mRNA stability. To generate reactive oxygen species within susceptible macrophages, menadione was added to macrophage cultures 30 min before the addition of actinomycin D. Menadione is a recycling quinone; the recycling is catalysed by intracellular reductases forming a semiquinone free radical that on reaction with molecular oxygen releases a superoxide

Figure 5 PKC inhibitors inhibited Nramp1 mRNA stability in Nramp1^{Gly169} macrophages

RAW264.7 macrophages expressing Nramp1^{Gly169} or Nramp1^{Asp169} were infected with *M. avium* for 16 h. PKC inhibitors bisindolylmaleimide I (Bis; 20 μ M) and Gö6976 (1 μ M) were added 30 min before the addition of actinomycin D. RNA was isolated at 0, 3, 6, 9 and 12 h after the addition of actinomycin D. (A, B) Representative Northern blots: (A) Nramp1^{Gly169}; (B) Nramp1^{Asp169}. Numbers under Nramp1 blots are Nramp1 mRNA levels relative to zero time determined by a densitometric analysis of the Nramp1 and G3PDH blots and calculated as described in the legend to Figure 1. (C, D) Nramp1 mRNA decay curves derived from Northern blot hybridizations are shown for Nramp1^{Gly169} macrophages (C) and Nramp1^{Asp169} macrophages (D). Symbols: ●, control; ○, bisindolylmaleimide I; ▼, Gö6976. Results are means $\pm$ S.E.M. for three separate experiments. (E) Nramp1 mRNA half-lives derived from the decay curves. Results are means $\pm$ S.D. for the three experiments. Bisindolylmaleimide I and Gö6976 significantly inhibited Nramp1 mRNA half-lives in Nramp1^{Gly169} macrophages ($P < 0.005$, Student's *t* test).

anion and regenerates the menadione molecule [32]. Further metabolism of the superoxide anion generates a variety of active oxygen species, such as H₂O₂ and hydroxyl radicals. As shown in Figures 6(A) and 6(B), menadione increases Nramp1 mRNA stability in Nramp1^{Asp169} macrophages. The half-life of Nramp1 mRNA was increased from 10.4 $\pm$ 0.6 to 29.8 $\pm$ 4.2 h (Figure 6C). Menadione treatment of Nramp1^{Gly169} macrophages also increased Nramp1 mRNA stability, resulting in mRNA that was stable for 12 h (results not shown). To determine whether PKC activity is required for the increased Nramp1 stability, the PKC inhibitor Gö6976 was added to cultures of Nramp1^{Asp169} macrophages 1 h before the addition of menadione. The pre-treatment of macrophages with the PKC inhibitor completely blocked the effects of menadione on Nramp1 mRNA stability.

PKC activity in *M. avium*-infected Nramp1^{Gly169} macrophage is inhibited by antioxidants

We examined the PKC activity of the particulate fraction of *M. avium*-infected Nramp1^{Gly169} and Nramp1^{Asp169} macrophages (Figure 7A). The PKC activity of Nramp1^{Gly169} macrophages increased after infection and was still elevated at 4 h after infection. In contrast, the PKC activity of Nramp1^{Asp169} macrophages increased slightly at 1 h and quickly declined to background. To determine whether the generation of reactive oxidants is involved in the activation of PKC, macrophages were infected with *M. avium* in the presence of the antioxidant BHA (Figure 7B). The antioxidant inhibited the increased PKC activity of Nramp1^{Gly169} macrophages induced by *M. avium* infection. To

Figure 6 Generation of reactive oxygen species in *Nramp1*^{Asp169} macrophages increases *Nramp1* mRNA stability and this increase in mRNA stability is blocked by inhibiting PKC activity

RAW264.7 macrophages expressing *Nramp1*^{Asp169} were incubated with *M. avium* for 16 h. Menadione (50 μ M) was added 30 min before the addition of actinomycin D. To block the effect of menadione, the protein kinase inhibitor Gö6976 (1 μ M) was added 1 h before the addition of menadione. RNA was isolated 0, 3, 6, 9 and 12 h after the addition of actinomycin D. (A) Representative Northern blots hybridized with *Nramp1* and *G3PDH* probes. Numbers under *Nramp1* blots are *Nramp1* mRNA levels relative to zero time determined by a densitometric analysis of the *Nramp1* and *G3PDH* blots and calculated as described in the legend to Figure 1. (B) *Nramp1* mRNA decay curves derived from a densitometric analysis of the Northern blots. Symbols: ●, control; ○, menadione; ▼, menadione plus Gö6976. Results are means $\pm$ S.E.M. for three separate experiments. (C) *Nramp1* mRNA half-lives derived from the decay curves. Results are means $\pm$ S.D. for the three experiments. Treatment of macrophages with menadione significantly increased the *Nramp1* mRNA half-life compared with control untreated macrophages ($P < 0.005$). This increase in mRNA stability was blocked by the PKC inhibitor ($P < 0.005$).

explore further the role of oxidants in PKC activation, we examined the effect of menadione generation of superoxide anions on PKC activity. As shown in Figure 7(C), menadione treatment increased PKC activity in both *Nramp1*^{Gly169} and *Nramp1*^{Asp169} macrophages. Menadione and *M. avium* induced an equal level of PKC activity in *Nramp1*^{Gly169} macrophages, suggesting that PKC is equally activated by reactive oxygen species resulting from *Nramp1*-mediated iron transport and reactive oxygen species generated by menadione treatment.

DISCUSSION

Nramp1, an iron-transport protein that localizes to the phagosomes of macrophages, has been shown to mediate host resistance by transporting iron into the phagosome, where the iron catalyses the formation of hydroxyl radicals via the Fenton/Haber–Weiss reaction [19,20]. In addition to its role in mediating anti-microbial

activity, *Nramp1* has been reported to have more pleiotrophic effects by controlling the functional capacity of macrophages [21–26]. For example, the expression of MHC class II glycoproteins is more stable in macrophages from *Nramp1* wild-type mice and these macrophages also produce greater quantities of inflammatory cytokines. We have previously suggested that this increase in cytokine production, increase in stability of MHC class II expression and increase in production of anti-microbial effector molecules might be the result of an increase in mRNA stability [8,27]. The mRNA half-lives of tumour necrosis factor α , inducible nitric oxide synthase and MHC class II E β chain, as well as *Nramp1*, was greater in macrophages from *Nramp1*^{Gly169} mice than those in macrophages from *Nramp1*^{Asp169} mice. In the present study we showed that the expression and stability of *Nramp1* mRNA are higher in RAW264.7 cells transfected with *Nramp1*^{Gly169} than in the same cell line transfected with *Nramp1*^{Asp169}. We have also observed that the stability of mRNA

Figure 7 PKC activity induced by *M. avium* infection in *Nramp1*^{Gly169} macrophages is inhibited by an antioxidant

(A) *M. avium* infection induces greater PKC activity in *Nramp1*^{Gly169} macrophages (●) than in *Nramp1*^{Asp169} macrophages (○). Macrophages were infected with *M. avium* for 0, 1, 2 and 4 h. Cells were harvested and lysed, and PKC activity was determined in the protein particulate fraction after DE52 chromatography. (B) Treatment with the antioxidant BHA inhibits the PKC activity induced by *M. avium* infection in *Nramp1*^{Gly169} macrophages. Macrophages were infected with *M. avium* (hatched columns) or *M. avium* in the presence of BHA (250 μM) (grey columns). After 3 hours, the PKC activity of the particulate fraction was determined. Open columns, unstimulated. (C) Generation of oxidants with menadione induces PKC activity in both *Nramp1*^{Gly169} and *Nramp1*^{Asp169} macrophages. Macrophages were infected with *M. avium* (hatched columns) or treated with menadione (50 μM) (grey columns). After 3 h the PKC activity of the particulate fraction was determined. Open columns, unstimulated. Results are means ± S.E.M. for triplicate measurements. Results of one representative experiment of three are shown.

from two IFN-γ-inducible genes, *TGTP* and *GBP-1*, which encode intracellular guanylate-binding proteins [29,33,34], is higher in IFN-γ-stimulated *Nramp1*^{Gly169}-transfected RAW264.7 cells than in *Nramp1*^{Asp169}-transfected RAW264.7 cells (W. P. Lafuse and G. R. Alvarez, unpublished work). These differences in mRNA stability were observed in macrophages that had been activated by *M. avium* or IFN-γ. Whether differences in mRNA stability exist in unstimulated macrophages has not been addressed. The levels of *Nramp1*, *TGTP* and *GBP-1* mRNA in unstimulated cells are not sufficient to permit accurate mRNA decay measurements. We are currently cloning regions of the *Nramp1* gene, in particular the 5' untranslated region (UTR), into a luciferase expression vector. Besides identifying *Nramp1* sequences responsible for mRNA stability, we shall be able to compare mRNA stability in unstimulated and activated macrophages.

We have previously shown that the transport of iron *in vivo* into phagosomes containing *M. avium* is greater in *Nramp1*^{Gly169}-transfected than in *Nramp1*^{Asp169}-transfected RAW264.7 macrophages [19]. In the present study we showed that the removal of available iron by chelation with desferrioxamine decreased the level of *Nramp1* mRNA stability in *Nramp1*^{Gly169} macrophages to the level observed in *Nramp1*^{Asp169} macrophages. This observation differs from that in our previous study [20] in which desferrioxamine treatment of IFN-γ-activated splenic macrophages from *Nramp1* resistant and susceptible mice resulted in an increased mRNA stability in the susceptible strains. There is a major difference between the two studies that might account for this difference. First, the iron chelator was added at the same time as IFN-γ; *Nramp1* mRNA stability was determined 20 h later. Thus iron chelation might be affecting the expression of genes regulated by intracellular iron levels. In the present study, the iron chelator, as well as antioxidants and PKC inhibitors, were added 30 min before the addition of actinomycin D so that there is no effect on gene expression. Secondly, the studies differ in their modes of macrophage activation, IFN-γ compared with *M. avium* infection. A differential effect of iron chelation on *Nramp1* protein expression has been reported by Atkinson et al. [10] in which they showed that iron chelation increased *Nramp1* protein induced by IFN-γ, whereas iron chelation diminished *Nramp1* protein expression in macrophages stimulated with both LPS and IFN-γ.

One consequence of the mobilization of iron and the catalysis of the Haber–Weiss reaction is the production of toxic intermediates of oxygen, including hydroxyl radicals. The response of cells to this oxidative stress is generally associated with the activation of signal transduction pathways by reactive oxygen species, resulting in the initiation of gene expression [35–37]. The results of this study suggest that another consequence of oxidant generation is the stabilization of mRNA. We found that antioxidants decreased the stability of *Nramp1* mRNA in macrophages expressing the resistant *Nramp1*^{Gly169} allele to the level observed for *Nramp1* mRNA stability in macrophages expressing the susceptible *Nramp1*^{Asp169} allele. The demonstration that an intracellular generator of superoxide anions also resulted in more stable mRNA in macrophages transfected with *Nramp1*^{Asp169} reinforces this interpretation. The effect is not limited to *Nramp1* mRNA stability because we have found that antioxidants also inhibit the stabilization of mRNA from *TGTP* and *GBP-1* in *Nramp1*^{Gly169}-transfected RAW264.7 macrophages (W. P. Lafuse and G. R. Alvarez, unpublished work).

Previous work [26,27] has shown that macrophages from *Nramp1*^{Gly169} congenic mice have higher levels of PKC activity than those from *Nramp1*^{Asp169} mice when activated either by IFN-γ or *M. bovis*. Similar differences in PKC activity were

observed in this study after infection with *M. avium*. Additionally, we showed that PKC inhibitors prevented the increase in mRNA stability after *M. avium* infection of *Nramp1^{Gly169}*-transfected macrophages. Similarly, the increased mRNA stability that followed the treatment of *Nramp1^{Asp169}*-transfected cells with an oxidant generator was prevented by inhibiting PKC activity. These results suggest that toxic oxygen intermediates stabilize Nramp1 mRNA by a PKC-dependent pathway that is activated by reactive oxygen species. We did not attempt to identify the PKC isoform responsible for the increased PKC activity in *M. avium*-infected *Nramp1^{Gly169}*-transfected macrophages. However, the stability of Nramp1 mRNA decreased after the addition of an inhibitor specific for the Ca²⁺-dependent isoforms of PKC (α , β and γ). Both PKC α and PKC β associate with the phagosome [38,39]. PKC α also regulates the innate function of macrophages involved in the control of infection by *Leishmania donovani* and *Legionella pneumophila* [40].

The stability of mRNA is regulated by *cis*-acting elements within the 3' UTR of mRNA that bind cytoplasmic *trans*-acting factors. The most studied of these *cis*-acting elements is an A+U-rich element present in the 3' UTR of lymphokine genes and proto-oncogenes *c-myc* and *c-fos* [41,42]. mRNA species that express this *cis*-acting element in their 3' UTR are rapidly degraded in the cytoplasm. There is also evidence that lymphoid cells can regulate the stability of lymphokine mRNA that express this element [42–45]. These studies showed that treatment with phorbol esters, calcium ionophores and interleukins increase the stability of lymphokine mRNA. However, *Nramp1* does not contain an A+U-rich element and the stability of Nramp1 mRNA is much greater than genes that contain A+U-rich elements. Another candidate element that also controls mRNA stability is the iron response element. Iron response elements are stem loop motifs present in the 5' UTR of ferritin transcripts and the 3' UTR of the transferrin receptor mRNA [46–48]. Iron regulatory proteins bind to the iron response elements when iron levels are low, and act as a repressor of ferritin translation and stabilize transferrin mRNA [49–52]. When iron levels are high, the iron regulatory proteins bind iron and release from the mRNA. However, the *Nramp1* 3' UTR does not contain an iron response element.

What, then, accounts for the changes in mRNA stability that we have observed? Phagocytosis of *M. avium* results in the recruitment of Nramp1 to the phagosome. The acidic environment results in the transport of iron into the phagosome. Iron catalyses the production of hydroxyl radicals via the Haber–Weiss reaction. The generation of oxidants activates PKC-dependent pathways that result in the activation of *trans*-acting factors that interact with Nramp1 mRNA and stabilize the mRNA. This activation is likely to occur through the phosphorylation of the *trans*-acting factors. Although PKC might directly phosphorylate the *trans*-acting factors, PKC can act upstream of mitogen-activated protein (MAP) kinase signalling cascades. A role for extracellular signal-regulated kinases (ERK1 and ERK2) and p38 in the regulation of Nramp1 levels is suggested by studies of Zhang et al. [53], who showed that inhibitors of ERK1, ERK2 and p38 MAP kinase decrease the expression of pig Nramp1 mRNA. We have also found that inhibitors of ERK1, ERK2 and p38 MAP kinase activation inhibit Nramp1 mRNA stability in *Nramp1^{Gly169}* macrophages (W. P. Lafuse and G. R. Alvarez, unpublished work).

The 3' UTR of Nramp1 mRNA does not contain consensus sequences that suggest that any of the known mRNA-binding elements are involved in the increased stability of the mRNA. However, we have found that *M. avium* infection induces protein binding to the *Nramp1* 3' UTR that is greater in *Nramp1^{Gly169}*-

transfected macrophages than in *Nramp1^{Asp169}*-transfected cells as assessed by RNA electrophoretic mobility-shift assays (W. P. Lafuse and G. R. Alvarez, unpublished work). The identities of these proteins and their requirement for phosphorylation are currently being investigated.

We thank Jenefer Blackwell for generously supplying the transfected macrophage cell lines. This work was supported by grants HL59795, AI42901 and MH54966 from the National Institutes of Health.

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Received 14 April 2000/2 August 2000; accepted 22 August 2000