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The *Bcg*/*Ity*/*Lsh* Locus: Genetic Transfer of Resistance to Infections in C57BL/6J Mice Transgenic for the *Nramp1*^{Gly169} Allele

GREGORY GOVONI,[1] SILVIA VIDAL,[2] SUSAN GAUTHIER,[1] EMIL SKAMENE,[2] DANIELLE MALO,[2] AND PHILIPPE GROS[1,2]*

Department of Biochemistry, McGill University,[1] and Center for the Study of Host Resistance, Montreal General Hospital Research Institute,[2] Montreal, Canada

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The murine *Bcg*/*Ity*/*Lsh* locus determines the susceptibilities of inbred strains to infection with unrelated intracellular parasites, such as *Mycobacterium bovis*, *Salmonella typhimurium*, and *Leishmania donovani*. A candidate for *Bcg*/*Ity*/*Lsh*, designated *Nramp1*, has been recently identified and shown to encode a novel integral membrane protein that is expressed exclusively in professional phagocytes but whose function remains unknown. In inbred strains, the susceptibility to infection is associated with a single glycine-to-aspartic acid substitution at position 169 (G169D) in the predicted TM4 of the protein. To confirm the candidacy of *Nramp1* as *Bcg*/*Ity*/*Lsh* and to determine the importance of the G169D mutation on Nramp1 function, we constructed transgenic mice in which the *G169* allele of *Nramp1* was transferred onto the background of a homozygous *D169* allele. These transgenic mice were analyzed for their sensitivity to infections under the control of *Bcg*/*Ity*/*Lsh*. The transgene constructed for these studies contained the entire *Nramp1*^{G169} gene together with approximately 5 kb of sequences upstream of the transcription initiation site of this gene. We observed that these sequences were sufficient to direct *Nramp1*^{G169} expression in transgenic macrophages, resulting in the appearance of a mature protein of 90 to 100 kDa over a background of *Nramp1*^{D169} characterized by the complete absence of the mature Nramp1 polypeptide. The appearance of the *Nramp1*^{G169}-encoded protein in transgenic macrophages was concomitant with the emergence of resistance to infection by *M. bovis* BCG, as measured by the extent of bacterial replication in the spleen, and by *S. typhimurium*, as measured by survival after an intravenous challenge. The gain of function detected in transgenic *Nramp1*^{G169} animals establishes unambiguously that *Nramp1* and *Bcg*/*Ity*/*Lsh* are allelic.

In the mouse, natural resistance or susceptibility to infection by a number of intracellular parasites, such as *Mycobacterium bovis*, *Mycobacterium intracellulare*, *Mycobacterium leprae*, *Salmonella typhimurium*, and *Leishmania donovani*, is determined by the chromosome 1 locus *Bcg*/*Ity*/*Lsh* (4, 24, 27, 28). The gene has two allelic forms (resistant, *Bcg*^{r}; susceptible, *Bcg*^{s}) in inbred strains and regulates the growth of these antigenically unrelated parasites in the reticuloendothelial (RE) organs during the early phase of infection (16). Studies in vivo (17) and experiments in vitro (8, 9, 14, 19, 29) using explanted cell populations have indicated that the macrophage is the most likely cell expressing the genetic difference at *Bcg*. Recent experiments with replication-defective variants of *S. typhimurium* strongly suggest that the resistance phenotype is associated with enhanced bacteriostatic activity rather than superior bactericidal activity of *Bcg*^{r} macrophages in vivo (3, 12). Using a positional cloning approach based on genetic and physical mapping, we have identified a candidate gene for *Bcg* (32). This gene, designated *Nramp1* (for natural resistance-associated macrophage protein 1), was found to be expressed exclusively in RE organs and in macrophages derived from them. It encodes a novel highly hydrophobic integral membrane protein, with 12 putative transmembrane domains, a glycosylated loop, several predicted phosphorylation signals for protein kinases A and C, a possible SH3 binding domain in the amino terminus (2), and a sequence motif that resembles a consensus transport signature found in several prokaryotic and eukaryotic membrane transport proteins (1). In addition, the Nramp protein defines a new family of membrane proteins that have been highly conserved in the evolution of eukaryotes (from yeasts to humans) and that share structural features common to ion channels and transporters (5). Recent studies of human blood cells and the promyelocytic leukemia HL-60 cell line have shown that *NRAMP1* is a specific marker of the myeloid lineage and is expressed at high levels in monocytes/macrophages as well as in polymorphonuclear leukocytes (6). Although the precise biochemical function of the Nramp1 protein remains unknown, it is believed to participate in a key bacteriostatic mechanism common to professional phagocytes of the monocytic and granulocytic lineages.

Sequence analysis of *Nramp1* in 27 inbred mouse strains of either *Bcg*^{r} or *Bcg*^{s} genotype showed that susceptibility to infection was associated with a single glycine-to-aspartic acid substitution at amino acid position 169 within the predicted TM4 of the protein (23). The nonconservative replacement of a small neutral glycine (G-169) by a negatively charged aspartate (D-169) in a predicted transmembrane domain was proposed to affect either proper targeting and folding of the protein in the membrane or to directly interfere with the enzymatic activity of Nramp1. Haplotype mapping studies using sequence polymorphisms identified within *Nramp1* and additional markers from this region revealed that although the 20 *Bcg*^{r} strains analyzed showed diverse allelic combinations for these markers, the 7 *Bcg*^{s} strains tested shared a conserved core haplotype of 2.2 Mb overlapping and including *Nramp1*

* Corresponding author. Mailing address: Department of Biochemistry, McGill University, 3655 Drummond St., Montreal, Quebec, Canada H3G-1Y6. Phone: (514) 398-7291. Fax: (514) 398-2603. Electronic mail address: gros@medcor.mcgill.ca.

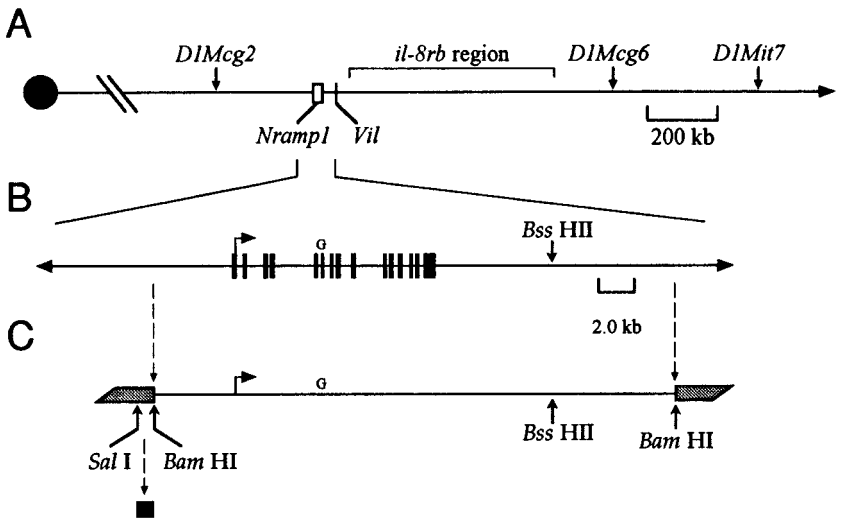

FIG. 1. Genomic organization of the *Nramp1* locus on mouse chromosome 1. (A) *Nramp1* has been mapped to the proximal portion of mouse chromosome 1. The positions of the *Vil* gene and dinucleotide repeat markers *D1Mcg2*, *D1Mcg6*, and *D1Mit7* are shown relative to that of *Nramp1* (22, 23). The physical location of the *il-8rb* gene is unknown, but linkage analysis places it roughly between *Nramp1* and the *D1Mcg6* marker (7, 30). (B) The genomic structure of *Nramp1* is shown. The distinguishing features of this gene include the translation initiation site within exon I (raised arrow), the glycine residue at position 169 (G) in exon VI, and the *Bss*HII restriction site 6 kb downstream of the last exon (exon XV). (C) The screening of a 129sv mouse strain (Bcg^r) genomic library yielded clone cosnramp.1. Nucleotide sequencing of this clone identified the presence of a Gly codon for residue 169. Restriction endonucleases *Sal*I and *Bss*HII were used to release a 23-kb DNA fragment used for microinjection. The pcos4 vector DNA included in the 23-kb DNA fragment was used as a hybridization probe to identify transgenic animals (black box) and was isolated as a 200-bp *Sal*I-to-*Bam*HI restriction fragment.

(23). Taken together, these results suggest that (i) G-169 is the wild-type form of *Nramp1* and that the nonconservative substitution of D-169 underlies the Bcg^s phenotype and that (ii) Bcg^s alleles carry the same G-169→D (G169D) mutation and are identical by descent.

To establish that *Bcg*/*Ity*/*Lsh* and *Nramp1* are indeed the same gene, we took two complementary experimental approaches. Firstly, we used homologous recombination in embryonal stem cells to disrupt the $Nramp1^{G169}$ allele of the 129sv strain (Bcg^r), which created a null mutation ($Nramp1^{-/-}$) at *Nramp1* (31). The targeting construct used in these studies caused an intragenic deletion in *Nramp1*, resulting in the absence of a full-length RNA and mature protein synthesized in mutant mice. $Nramp1^{-/-}$ mutants developed normally, were healthy, and showed a longevity similar to that of controls. However, an analysis of the kinetics of *M. bovis* BCG infection in the RE organs of these mice indicated that disruption of *Nramp1* abrogated the natural resistance of 129sv mice to BCG infection and also eliminated natural resistance to both *L. donovani* and lethal *S. typhimurium* infections. Studies of this loss-of-function mutant therefore strongly suggested that *Nramp1*, *Bcg*, *Lsh*, and *Ity* are indeed the same locus. In parallel, we carried out the reverse experiment (gain-of-function mutation) and used cloned genomic DNA corresponding to the $Nramp1^{G169}$ allele to transfer the resistance phenotype to an otherwise susceptible genetic background (Bcg^s). Here we report the creation and analysis of a transgenic mouse which carries a cloned $Nramp1^{G169}$ allele derived from the 129sv strain (which includes the gene and additional 5′ sequences) introduced and expressed in the context of a chromosome 1 genetic background (Bcg^s) donated by the C57BL/6J strain. The transfer of the $Nramp1^{G169}$ construct resulted in abundant synthesis of the corresponding Nramp1 protein in transgenic macrophages isolated from these mice and was concomitant with the appearance of resistance to infection by *M. bovis* BCG and *S. typhimurium*.

MATERIALS AND METHODS

Creation and characterization of transgenic mice. A genomic cosmid clone (cosnramp.1) containing the entire mouse *Nramp1* gene and 5 kb of 5′ sequences upstream of the major transcription initiation site was isolated from a genomic library constructed in cosmid vector pcos4 by using genomic DNA from mouse strain 129sv. This strain has the Bcg^r genotype and carries the Gly-169 allele of the *Nramp1* gene ($Nramp1^{G169}$) (15). A 23-kb fragment of this cosmid containing the 15 *Nramp1* genomic exons and 5 kb of 5′ sequences together with an additional 3′ sequence, including a polyadenylation signal, was excised from clones with restriction enzymes *Sal*I and *Bss*HII (Fig. 1). This fragment also contains approximately 200 bp of vector sequences derived from one of the pcos4 cosmid cloning arms (*Sal*I-to-*Bam*HI fragment; Fig. 1). The cosnramp.1 *Sal*I-to-*Bss*HII fragment was gel purified and resuspended in sterile distilled, deionized water. DNA (40 nl at 1 mg/ml) was microinjected in male pronuclei of fertilized eggs from a (C3H [Bcg^r/$Nramp1^{G169}$] × C57BL/6 [Bcg^s/$Nramp1^{D169}$]) hybrid obtained 0.5 days postcoitus. Injected eggs were then reimplanted in the uteri of pseudopregnant CD1 outbred female mice. Offspring positive for the transgene were identified by Southern blotting analysis of tail DNA, using vector sequences as the hybridization probe. They were subsequently backcrossed to C57BL/6 (Bcg^s/$Nramp1^{D169}$) to identify animals positive for the transgene ($Nramp1^{G169}$) and homozygous for C57BL6/J markers on the portion of chromosome 1 carrying *Nramp1*. These animals were then intercrossed to generate the $Nramp1^{G169}$ transgenic line on the homozygous $Nramp1^{D169}$/$Nramp1^{D169}$ chromosome 1 background. Inbred mice were purchased from the Jackson Laboratories (Bar Harbor, Maine) and subsequently maintained in our animal quarters.

Genomic DNA analysis. Total genomic DNA was extracted from tail biopsies of individual 6-week-old mice by treatment with proteinase K (100 μg/ml at 55°C for 16 h) and RNase A (5 μg/ml). Genomic DNA was purified by successive phenol and chloroform extractions and precipitation with isopropyl alcohol, as previously described (23). To identify transgenic animals, genomic DNA was digested to completion with restriction enzyme *Bam*HI, separated by electrophoresis in 1% agarose gels containing TAE buffer (40 mM Tris-acetate, 20 mM sodium acetate, 20 mM EDTA [pH 7.6]), and transferred onto nylon hybridization membranes (Hybond-N; Amersham International, Little Chalfont, United Kingdom) by capillary blotting in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Southern blots were prehybridized for 16 h at 42°C and then hybridized for 16 h at 42°C in the same mixture composed of 50% formamide, 5× SSC, 1% sodium dodecyl sulfate (SDS), 10% dextran sulfate, 20 mM Tris (pH 7.5), 1× Denhardt's solution (0.1% bovine serum albumin [BSA], 0.1% Ficoll, 0.1% polyvinylpyrrolidone), and heat-denatured salmon sperm DNA at a final concentration of 200 μg/ml. The pcos4 hybridization probe was a 200-nucleotide *Sal*I-to-*Bam*HI fragment derived from one of the cosmid cloning arms, labeled to high-level specific activity (10^9 cpm/μg of DNA) by random priming (13) with [α-^{32}P]dATP (specific activity, 3,000 Ci/mmol), and used at 10^6

cpm/ml of hybridization solution. The membranes were washed to a final stringency of 0.1× SSC–0.1% SDS at 65°C for 30 min. Autoradiography of all hybridized filters was done on Kodak XAR film at −80°C with an intensifying screen. Simple sequence length polymorphism markers *D1Mcg2*, *D1Mcg6*, and *D1Mit7* (23) were used to determine the allelic combination of loci mapping in the proximity of *Nramp1* on chromosome 1 among the offspring of *Nramp1*^{G169} transgenic animals. For PCR amplification of simple sequence length polymorphisms, a 20-ng aliquot of genomic DNA dissolved in PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 9.0], 1.5 mM $MgCl_2$, 0.1% Triton X-100) was used in a 50-μl PCR after the addition of sequence-specific oligonucleotide primers (100 nmol), deoxynucleoside triphosphates (200 μM each) and 1 U of *Taq* DNA polymerase (BIOCAN, Montreal, Canada). The thermocycling program consisted of one initial denaturation at 94°C for 3 min and 30 three-step cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by a final cycle at 72°C for 7 min. One of the two oligonucleotide primers was labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase, and 20 nmol of the ^{32}P-labeled primer was added to the PCR mixture. ^{32}P-labeled PCR products were diluted twofold in 100% formamide, denatured for 5 min at 90°C, and electrophoresed in denaturing 8% polyacrylamide gels containing 8 M urea. Gels were dried and exposed to Kodak XAR film for 4 to 12 h.

Experimental infections in mice. The infectious inoculum of *M. bovis* BCG (strain Montreal) was prepared as described previously (16). *Bcg*^{r} and *Bcg*^{s}-control mice, together with transgenic animals (a minimum of four per group), were inoculated in the caudal tail vein with 0.25 ml of physiological saline containing 2×10^4 CFU of BCG. Three weeks after infection, mice were sacrificed by cervical dislocation and their spleens were removed under aseptic conditions to determine the number of BCG CFU. Briefly, the spleens were weighed and homogenized by using sterile mortars and pestles with 90-mesh alumdum and 5 ml of physiological saline containing 0.5% BSA fraction V (Sigma). Spleen homogenates were further diluted in the same buffer and plated onto Dubos solid medium (Difco) for the determination of viable bacilli, as previously described (16). The number of BCG CFU was determined after a 3-week incubation of bacterial plates at 37°C, and the geometric mean was calculated. Highly virulent *S. typhimurium* Keller was originally obtained from Hugh Robson (Royal Victoria Hospital, Montreal, Canada). A bacterial culture was grown overnight in tryptic soy broth (diluted in phosphate-buffered saline), and serial dilutions were plated onto L agar with subsequent incubation at 37°C for 24 h to establish the CFU count of the overnight culture. Mice (groups of four or five animals) were injected intravenously (caudal tail vein) with a 0.2-ml inoculum containing 4.5×10^4 CFU of *S. typhimurium* per ml. Animals were examined twice a day for up to 4 weeks, deaths were recorded, and moribund animals were sacrificed. By using this protocol, susceptible (*Ity*^{s}) animals typically succumbed to infection within 4 to 6 days while resistant animals (*Ity*^{r}) typically survived at least 14 days postinfection (31).

Metabolic labeling and immunoprecipitation. Thioglycolate-elicited peritoneal macrophages from control BALB/cJ (*Bcg*^{s}), 129sv (*Bcg*^{r}), and C57BL/6 *Nramp1*^{G169} transgenic mice were harvested 4 days after injection by peritoneal lavage with 10 ml of Hanks' balanced salt solution. Peritoneal cells were allowed to adhere to plastic for a minimum of 4 h (37°C, 5% CO_2 atmosphere) in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum, 3 mM glutamine, penicillin (50 U/ml), and streptomycin (50 μg/ml). Nonadherent cells were removed, adherent cells were washed once with methionine-free RPMI medium, and the medium was replaced with methionine-free RPMI medium containing 10% dialyzed fetal bovine serum, 5 mM glutamine, and [^{35}S]methionine (1,000 Ci/mmol; New England Nuclear) at a final concentration of 50 μCi/ml of culture medium. Labeling was then allowed to proceed by further incubation for a minimum of 12 h at 37°C. Cells were harvested with a plastic scraper, rinsed once in cold phosphate-buffered saline, and lysed in a buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 0.2% SDS, and 1% Triton X-100 (26). For immunoprecipitation of the Nramp1 polypeptide, we generated a rabbit hyperimmune antiserum (GST-54N) directed against a glutathione *S*-transferase fusion protein containing the first 54 amino-terminal residues of Nramp1 (33). Individual labeled cell extracts (10^7 cpm) were incubated with the GST-54N antibody (used at a 1:100 dilution) for 16 h at 4°C, and specific immune complexes were isolated by further incubation with a 50:50 mixture of protein A and protein G coupled to Sepharose beads (Pharmacia) for 2 h at 4°C (26). The beads were then washed extensively in a buffer containing 150 mM NaCl, 0.1% Triton X-100, and 0.03% SDS. Finally, the samples were resuspended in Laemmli sample buffer and electrophoresed on an SDS–7.5% polyacrylamide gel. Fluorography of the SDS-polyacrylamide gel electrophoresis (PAGE) gel was performed with a commercially available amplifier (En^{3}Hance; Du Pont) according to the recommendations of the manufacturer. The gel was dried and exposed for 24 h at −80°C.

RESULTS

We previously characterized the genomic organization of the mouse *Nramp1* gene, including the delineation of individual exons and the mapping of the major transcription initiation sites (15). This gene is composed of 13 coding and 2 untranslated exons, spanning a total of 12 kb on the proximal portion of mouse chromosome 1 (Fig. 1). To attempt genetic transfer of the phenotype for resistance to infection encoded by *Bcg* with a cloned *Nramp1* copy, we isolated a *Nramp1* cosmid clone (cosnramp.1) from a genomic library constructed in cosmid vector pcos4 with genomic DNA from the 129sv mouse strain (*Bcg*^{r}/*Nramp1*^{G169}). cosnramp.1 was found to contain the entire *Nramp1* gene together with additional 5′ and 3′ flanking sequences (Fig. 1). A 23-kb *Sal*I-to-*Bss*HII restriction fragment from this cosmid containing (from 5′ to 3′) approximately 200 nucleotides of pcos4 sequences, 5 kb of 5′ sequences located upstream of the major *Nramp1* transcription start site, the entire structural gene (15 exons), and 6 kb of 3′ downstream sequences was gel purified and injected in male pronuclei of (C3H × C57BL/6)F_1 fertilized eggs (*Nramp1*^{G169}/*Nramp1*^{D169} heterozygotes). These eggs were reimplanted in pseudopregnant CD1 females, and transgenic pups were identified among the offspring by Southern blotting analysis of tail DNA, using the 200-nucleotide *Sal*I-to-*Bam*HI pcos4 arm fragment representing the extreme 5′ end of the injected DNA fragment as a hybridization probe (Fig. 2). As expected, this pcos4 DNA probe did not detect any hybridizing fragment in normal mouse DNA but identified unambiguously transgenic animals that had received the corresponding cosmid DNA fragment (Fig. 2). Among 50 offspring tested, three founder animals (3855, 5569, and 5585) were identified.

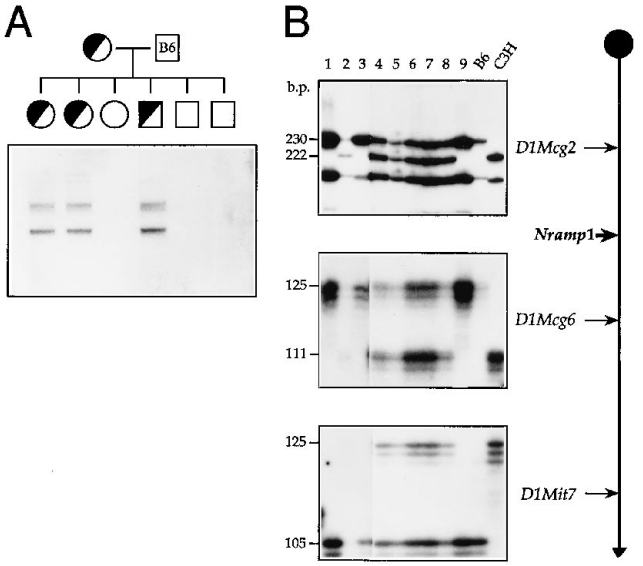

FIG. 2. Genotyping of transgenic animals. (A) The founder (female 3885; semiblackened circle) of strain (C3H/HeJ × C57BL/6J)F_1 was crossed with a C57BL/6J male (square). The resulting progeny (a sample of six is shown) were tested for the presence of the transgene (*Nramp1*^{*G169}) by Southern blotting. Mice positive for the transgene (semiblackened circles or squares) show genomic *Bam*HI fragments of approximately 4.8, 6.4, and 6.5 kb that hybridize to the 200-bp pcos4 vector probe. (B) Examples of genotyping with mouse chromosome 1 markers are shown. The diagram on the right represents the relative positions of dinucleotide repeat markers *D1Mcg2*, *D1Mcg6*, and *D1Mit7* to that of the *Nramp1* gene. The three panels show the respective PCR amplification products obtained for each marker with radiolabeled oligonucleotides. Shown are the results for a sample of progeny resulting from founder 3885 matings (lanes 1 through 9) or from control mouse strains C57BL/6J (lane 10) and C3H/HeJ (lane 11).

Since (i) the (C3H × C57BL/6)F_1 fertilized eggs used as recipients for the microinjected *Nramp1*^{G169} gene are heterozygotes for *Nramp1* alleles (*G169*/*D169*) and (ii) the *G169* (*Bcg*^{r}) resistance allele of C3H is dominant over the *D169* (*Bcg*^{s}) susceptibility allele of C57BL/6J, it was necessary to

isolate the transgenic *Nramp1*^{G169} allele onto a homozygous recessive *D169* susceptibility background in order to detect the possible appearance of resistance associated with this transgene. For this reason, three founder mice (3855, 5569, and 5585) were bred to C57BL/6 and offspring from this cross were first analyzed for the presence of this transgene (Fig. 2). The haplotypes of transgenic animals in these litters for chromosome 1 markers flanking the endogenous *Nramp1* gene on either side were determined by using one proximal (*D1Mcg2*) and two distal (*D1Mcg6* and *D1Mit7*) simple sequence length polymorphism markers (23) (Fig. 2). Upon PCR amplification, *D1Mcg2*, *D1Mcg6*, and *D1Mit7* produce genomic DNA diagnostic fragments of 230, 125, and 105 bp for C57BL/6 mice and 222, 111, and 125 bp for C3H mice, respectively (Fig. 2B). This analysis allowed us to distinguish between transgenic animals from this F_1 litter that were either heterozygotes (B6/C3) or homozygotes (B6/B6) for the three chromosome 1 B6 alleles tested. Male and female mice bearing the *Nramp1*^{G169} transgene on an otherwise *Nramp1*^{D169}/*Nramp1*^{D169} chromosome 1 background (B6/B6) were intercrossed to expand the transgenic line, and these individual F_2 animals were analyzed for the presence of this transgene by Southern blotting as described above. Transgenic mice appeared to be normal, developed normally, and showed fitness and longevity similar to those of nontransgenic littermates.

We recently developed a rabbit polyclonal antiserum against the Nramp1 polypeptide, which was raised against a fusion protein consisting of the first 54 N-terminal amino acid residues of Nramp1 fused in frame at the C terminus of glutathione *S*-transferase (GST-54N). By immunoprecipitation, this antiserum detected in macrophages derived from *Bcg*^{r} mice (*Nramp1*^{G169}) a specific heavily glycosylated and phosphorylated membrane protein of approximately 90 to 100 kDa (33). Using this antibody as an analytical tool, we were able to demonstrate that the G169D mutation characteristic of *Bcg*^{s} mice is characterized by a complete absence of the mature protein in macrophages, possibly through aberrant targeting and/or instability (33). We have taken advantage of this unique biochemical difference between the two allelic variants of the Nramp1 protein to identify the G169 protein product of the transgene in our F_2 animals. Briefly, thioglycolate-elicited peritoneal cells from control 129sv (*Bcg*^{r}/*Nramp1*^{G169}) and control BALBc/J (*Bcg*^{s}/*Nramp1*^{D169}) animals together with those from transgenic C57BL/6J mice (*Nramp1*^{G169} transgene) were harvested and metabolically labeled with [^{35}S]methionine. Then the Nramp1 protein was immunoprecipitated with the anti-Nramp1 antiserum GST-54N (33) and analyzed by SDS-PAGE (Fig. 3). In control 129sv macrophages (*Nramp1*^{G169}), this antiserum recognized specifically an abundant polypeptide of approximately 90 to 100 kDa (absent in the control preimmune serum lane) that migrated as a smear, which is typical of a heavily glycosylated protein. Parallel immunoprecipitation of the control BALBc/J cell extract (*Nramp1*^{D169}) failed to detect any specific Nramp1 protein. However, the introduction of a *Nramp1*^{G169} transgene on an otherwise *Nramp1*^{D169} background resulted in the appearance of a very abundant immunoreactive protein with an electrophoretic mobility and characteristics indistinguishable from those detected by the same serum in 129sv cells (Fig. 3). Together, these findings indicate that the transferred transgene is functional and capable of encoding a full-length G169 Nramp1 polypeptide. In addition, the transgenic construct used appears to include genomic sequences capable of directing macrophage expression, the normal site of expression of the *Nramp1* mRNA and protein.

The phenotypic consequences of G169 Nramp protein expression (in an otherwise D169 background) on macrophage function and resistance to infection were evaluated in transgenic animals for two infections under the control of *Bcg*/*Lsh*/*Ity*, *M. bovis* BCG and *S. typhimurium*. For BCG typing, control C57BL/6J (*Bcg*^{s}/*Nramp1*^{D169}), control C3H/HeJ (*Bcg*^{r}/*Nramp1*^{G169}), and C57BL/6J mice transgenic for the *Nramp1*^{G169} allele were infected with BCG (5×10^3 CFU) intravenously. The total spleen BCG CFU load was determined 3 weeks after infection (Fig. 4A). In control C57BL/6J mice, bacterial proliferation was rapid and uncontrolled in the early phase, reaching a maximum at 3 weeks (10^5 CFU of BCG per spleen). On the other hand, in control resistant C3H/HeJ animals, little or no bacterial proliferation was observed in the spleens of these mice at any time during infection (maximal load, 3.0×10^3 CFU of BCG per spleen at 3 weeks postinfection). At 3 weeks, the difference in the numbers of BCG CFU in spleens between the two groups was highly significant, at 100-fold ($P < 0.01$). Finally, mice from the C57BL/6J *Nramp1*^{G169} transgenic group showed a spleen bacteria load at 3 weeks after infection (3.8×10^3 CFU) which was indistinguishable from that observed in *Bcg*^{r} C3H/HeJ controls (Fig. 4A). These results show that transfer of the *Nramp1*^{G169} allele onto the otherwise susceptible genetic background of the C57BL/6J strain restores the capacity of these mice to control BCG growth in the spleen.

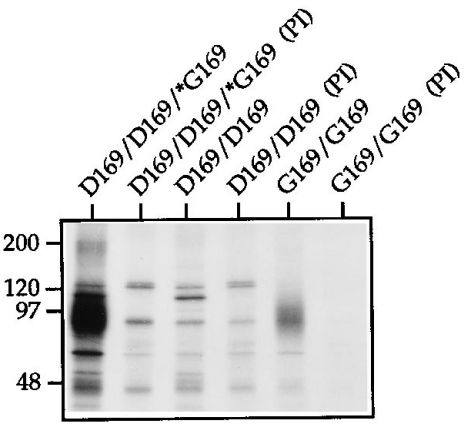

FIG. 3. Identification of the Nramp1 protein. [^{35}S]methionine-radiolabeled protein extracts from thioglycolate-elicited peritoneal macrophages were obtained from C57BL/6J transgenic (*Nramp1*$^{D169/D169/*G169}$), C57BL/6J (*Nramp1*$^{D169/D169}$), and 129sv (*Nramp1*$^{G169/G169}$) animals and subjected to immunoprecipitation with either normal (preimmune [PI]) serum or with GST-54N rabbit hyperimmune antiserum. The resulting immunocomplexes were separated by SDS-PAGE. The apparent molecular masses (in kilodaltons) of protein markers are on the left.

In contrast to infection by mycobacteria, *S. typhimurium* is a highly virulent mouse pathogen that replicates very rapidly in RE organs and produces an overwhelming and rapidly lethal infection in susceptible mouse strains. Other inbred mouse strains are completely resistant and survive an infectious challenge well; this difference is controlled by the *Bcg*/*Ity*/*Lsh* locus (24, 31). For *S. typhimurium* typing, control C57BL/6J (*Bcg*^{s}/*Nramp1*^{D169}), control 129sv (*Bcg*^{r}/*Nramp1*^{G169}), and C57BL/6J mice transgenic for the *Nramp1*^{G169} allele were infected with 0.9×10^4 CFU of *S. typhimurium* Keller intravenously (25) and survival to this inoculum was monitored over time. The results shown in Fig. 4B show that control C57BL/6J mice quickly succumbed to this infectious inoculum, with no animal from this group surviving more than 5 days. In contrast, all 129sv animals survived this period and were still healthy 3 weeks after infection (end of observation). On the other hand,

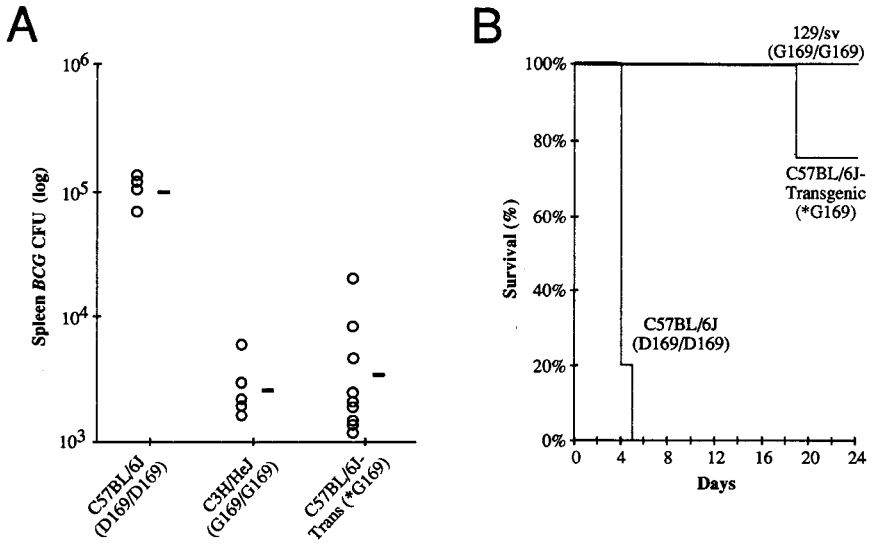

FIG. 4. Functional analysis of transgenic mice. (A) The spleens of mice infected intravenously with 5×10^3 CFU of BCG were removed 3 weeks after infection and homogenized. The BCG CFU in the spleens of individual mice (circles) were determined by plating serial dilutions of spleen homogenates on Dubos agar medium and counting the resulting CFU after 3 weeks. The results from individual C57BL/6J ($n = 4$), C3H/HeJ ($n = 5$), and C57BL/6J transgenic ($n = 9$) mice, together with the respective means (dashes), are shown. The CFU counts obtained for the control C57BL/6J susceptible strain were significantly different from those obtained for the control C3H/HeJ resistant and C57BL/6J transgenic strains (Student t test; $P < 0.01$), while CFU counts obtained for the two latter groups were not significantly different by the same analysis. (B) The survival of mice infected intravenously with 0.9×10^4 CFU of *S. typhimurium* was monitored for 24 days. C57BL/6J ($n = 5$), 129sv (*Nramp1*$^{G169/G169}$; $n = 4$), and C57BL/6J transgenic ($n = 4$) animals were examined twice a day to identify moribund and dead mice.

C57BL/6J mice transgenic for the *Nramp1*^{G169} allele largely survived bacterial challenge, with three of four mice tested still alive at 24 days postinfection. These results indicate that transfer of the *Nramp1*^{G169} transgene onto the innately susceptible genetic background of C57BL/6J mice causes the appearance of resistance to an intravenous lethal challenge of *S. typhimurium.*

Together, these results establish that *Nramp1* and *Bcg/Ity/Lsh* are the same gene and that sequences contained in the 5-kb region flanking the transcription initiation site are sufficient to direct proper *Nramp1* expression.

DISCUSSION

Despite the complete association of the G169D substitution with susceptibility to infection in inbred strains and the unfavorable structural context in which the mutation would be found (in the hydrophobic face of TM4), G169D did not constitute an obvious loss of function and direct experimental data needed to be obtained to certify (i) that *Nramp1* and *Bcg/Ity/Lsh* are indeed the same gene and (ii) that the G169D substitution is the formal cause of susceptibility to infection in *Bcg*^{s} mice. To tackle these important questions, we took two complementary experimental approaches. The first one was to create a null mutation at *Nramp1* by homologous recombination in embryonal stem cells and evaluate the effects of loss of *Nramp1* function on resistance to infections in vivo in *Nramp1*$^{-/-}$ mice (31). In these recent studies, we showed that the loss of *Nramp1* function results in the loss of natural resistance of 129sv mice (*Bcg*^{r}) to infection by *M. bovis*, *S. typhimurium*, and *L. donovani*, providing strong evidence that *Nramp1* and *Bcg/Ity/Lsh* are indeed allelic. One of the potential limitations of gene knockout experiments was that the targeting construct used introduced in this case a 4-kb deletion in the *Nramp1* gene. It is conceivable that this deletion, together with the insertion of a *neo* cassette with its own regulatory sequences, although clearly abrogating normal *Nramp1* mRNA production, may affect the overall chromatin structure in this chromosomal region and possibly alter regulation of the expression of other neighboring genes. In the case of *Nramp1*, this is of particular importance since the cellular receptor for interleukin-8 (*il-8rb*) maps extremely close to *Nramp1* and could also be affected by a targeting event (7, 30). This gene encodes the receptor for a major cytokine that plays a key role in inflammatory response and granulocyte function, and interestingly, *il-8rb* gene expression is tightly regulated in a tissue- and cell-specific fashion very similar to that of *Nramp1* (18). To circumvent these limitations, we carried out, in parallel, experiments in which we transferred a cloned copy of *Nramp1*^{G169} associated with resistance onto a *Nramp1*^{D169} genetic background and analyzed the effects of this transfer on resistance to infection. In the present study, we have shown that transfer of the *Nramp1*^{G169} allele onto a *Nramp1*^{D169} background causes a gain of function and results in the appearance of resistance to infection on an otherwise susceptible background. Together, the results of gene targeting experiments (31) and transgenic transfer of *Nramp1*^{G169} (this study) unequivocally establish that *Nramp1* and *Bcg/Ity/Lsh* are allelic and that the G169D mutation underlies the apparent loss of function detected in *Bcg*^{s} animals.

Immunoprecipitation studies using a specific anti-Nramp1 polyclonal antibody failed to detect a mature Nramp1 protein in macrophages from *Bcg*^{s} animals, in contrast to *Bcg*^{r} macrophages, in which a clear 90- to 100-kDa glycosylated protein could be easily detected (33) (Fig. 3). The reason for the absence of the mature protein associated with the G169D mutation is unknown, but it could be due to misfolding and/or aberrant targeting, with most of the protein accumulating in the Golgi or endoplasmic reticulum, resulting in degradation and apparent rapid turnover. Such a situation has previously been noted for mutants in other membrane proteins, such as CFTR (10, 11) and P-glycoprotein (20), and has been shown in the last case to be due to the loss of association with a molecular chaperon (21). Expression of the *G169* allele on a *D169*

background resulted in the appearance of the Nramp1 protein in transgenic macrophages and restoration of resistance to infection, indicating that the presence of the mutant protein does not per se prevent maturation, proper folding, and targeting of the wild-type protein to the appropriate membrane compartment of phagocytes. We have proposed that Nramp1 modifies the intracellular milieu of such a membranous compartment to influence microbial replication. Although the precise subcellular localization of Nramp1 remains to be clarified, we have proposed that it may be expressed in the phagosome membrane, as the three major classes of pathogens affected by *Bcg*/*Ity*/*Lsh* initially transit through this compartment and subsequently use independent strategies for intracellular survival. The anti-Nramp1 antibody is currently being used to establish the subcellular localization of this protein in professional phagocytes.

In the mouse, we have observed that *Nramp1* expression is limited to RE organs and is enriched in macrophages derived from them (32). We have also shown that macrophage cell lines J774 and RAW264.7 express *Nramp1* mRNA and that this expression can be further modulated by treatment with lipopolysaccharide and gamma interferon (15). The human *NRAMP1* counterpart is also expressed in blood monocytes and tissue macrophages, but very high levels of the mRNA are also found in polymorphonuclear leukocytes (6), a population not analyzed in the murine model. This suggests that *NRAMP1* may be a marker of the myeloid lineage and induced late in the differentiation program of granulocytes and monocytes, a proposition verified in studies with the HL-60 promyelocytic leukemic cell line, in which induction toward either the granulocytic or monocytic pathway resulted in robust induction of the *NRAMP1* mRNA (6). The successful transfer of the resistance phenotype using a cloned copy of the $Nramp1^{G169}$ allele, together with Nramp1 protein expression in the macrophages of transgenic animals, indicates that the transgenic construct used contains the genomic sequence information required for *Nramp1* mRNA expression in macrophages. Although we did not determine if the $Nramp1^{G169}$ transgene is expressed in additional ectopic sites in transgenic animals (negative for expression in normal mice), our findings indicate that the 5 kb of upstream sequences present in the transgenic construct are at least sufficient for macrophage expression. Nucleotide sequence analysis of a 1-kb segment upstream of the major transcription initiation site of *Nramp1* reveals the presence of several consensus sequences for the binding of transcription factors associated with either constitutive (AP-1 and Sp-1), myeloid cell-specific (PU.1), and cytokine-inducible (nuclear factor–IL-6 and gamma interferon response element) gene expression (15). Some of these sites have been conserved in the proximal promoter regions of the human, mouse, and chicken homologs of this gene. The transgenic model described here can be used with additional transgenic animals for in vivo studies of the functional relevance of such sites and corresponding *trans*-acting factors to *Nramp1* gene expression. Finally, the *Nramp1* regulatory sequences identified in this study can also be used to construct expression cassettes to direct the expression of mutant variants of *Nramp1* in macrophages in order to study the function of the key sequence motifs defining the Nramp protein family.

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