Cellular Prion Protein Promotes *Brucella* Infection into Macrophages

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Abstract

The products of the *Brucella abortus* virB gene locus, which are highly similar to conjugative DNA transfer system, enable the bacterium to replicate within macrophage vacuoles. The replicative phagosome is thought to be established by the interaction of a substrate of the VirB complex with macrophages, although the substrate and its host cellular target have not yet been identified. We report here that Hsp60, a member of the GroEL family of chaperonins, of *B. abortus* is capable of interacting directly or indirectly with cellular prion protein (PrP<sub>C</sub>) on host cells. Aggregation of PrP<sub>C</sub> tail-like formation was observed during bacterial swimming internalization into macrophages and PrP<sub>C</sub> was selectively incorporated into macropinosomes containing *B. abortus*. Hsp60 reacted strongly with serum from human brucellosis patients and was exposed on the bacterial surface via a VirB complex–associated process. Under in vitro and in vivo conditions, Hsp60 of *B. abortus* bound to PrP<sub>C</sub>. Hsp60 of *B. abortus*, expressed on the surface of *Lactococcus lactis*, promoted the aggregation of PrP<sub>C</sub> but not PrP<sub>C</sub> tail formation on macrophages. The PrP<sub>C</sub> deficiency prevented swimming internalization and intracellular replication of *B. abortus*, with the result that phagosomes bearing the bacteria were targeted into the endocytic network. These results indicate that signal transduction induced by the interaction between bacterial Hsp60 and PrP<sub>C</sub> on macrophages contributes to the establishment of *B. abortus* infection.

Key words: Hsp60 • type IV secretion • macropinocytosis • intracellular replication • brucellosis

Introduction

*Brucella* species are Gram-negative bacteria that cause brucellosis with pathological manifestations of arthritis, endocarditis, and meningitis as well as undulant fever in humans and abortion and infertility in numerous domestic and wild mammals (1). The bacterium is endemic in many developing countries and is responsible for large economic losses and chronic infections in humans (2). *Brucella* species are facultative intracellular pathogens that survive within a variety of cells, including macrophages. The virulence of these species and the establishment of chronic infection are thought to be due essentially to their ability to avoid the killing mechanisms within macrophages (3). However, the molecular mechanisms accounting for these properties are not understood completely.

Recent studies with nonprofessional phagocyte HeLa cells have confirmed these observations, showing that *Brucella* inhibits phagosome–lysosome fusion and transits through an intracellular compartment that resembles autophagosomes. Bacteria replicate in a different compartment, containing protein markers normally associated with the endoplasmic reticulum, as shown by confocal microscopy and immunogold electron microscopy (4, 5).

*Brucella* internalizes into macrophages by swimming on the cell surface with generalized membrane ruffling for several minutes, a process termed “swimming internalization,” after which the bacteria are enclosed by macropinosomes (6). In this period, the phagosomal membrane continues to
maintain a dynamic state. Lipid raft–associated molecules, such as glycosylphosphatidylinositol (GPI)*-anchored proteins, GM1 gangliosides, and cholesterol, have been shown to be selectively incorporated into macropinosomes containing *Brucella abortus*. In contrast, late endosomal marker lysosomal–associated membrane protein (LAMP)-1 and host cell transmembrane proteins are excluded from the macropinosomes. The disruption of lipid rafts on macrophages markedly inhibits the VirB-dependent macropinocytosis and intracellular replication (6). These results indicated that the entry route of *B. abortus* into the macrophages determined the intracellular fate of the bacteria that was modulated by lipid rafts (6, 7).

The operon coding for export mechanisms specializing in transferring a variety of multimolecular complexes across the bacterial membrane to the extracellular space or into other cells has been described (8). These complexes, named type IV secretion systems, are also found in *B. abortus* (*virB* genes; 9–11). This operon comprises 13 open reading frames that share homology with other bacterial type IV secretion systems involved in the intracellular trafficking of pathogens. Type IV secretion systems export three types of substrates: (a) DNA conjugation intermediates, (b) the multisubunit pertussis toxin, and (c) monomeric proteins including primase, RecA, the *Agrobacterium tumefaciens* VirE2 and VirF proteins, and the *Helicobacter pylori* CagA protein (8). However, the substrates of the VirB secretion system of *B. abortus* and the target of the effector in host cells remain undefined.

In this study, we investigated the effector protein secreted by the type IV secretion systems and its receptor on the host plasma membrane. Our results implied that heat shock protein Hsp60 of *B. abortus* had an effector-like function, which was expressed on the bacterial surface by the type IV secretion–associated manner. The cellular prion protein (PrP*^C*) was identified as a receptor for the Hsp60. This receptor–ligand interaction regulates the establishment of *B. abortus* infection.

### Materials and Methods

**Reagents.** Gentamicin, protein A–Sepharose 4B beads, and 4′,6-diamidino-2-phenylindole (DAPI) were obtained from Sigma-Aldrich. Ni-NTA agarose beads were obtained from Qiagen. Alexa Fluor 594-streptavidin, Cascade blue goat anti–rabbit IgG, and Texas Red goat anti–rat IgG were obtained from Molecular Probes, Inc. Rhodamine goat anti–rabbit or mouse IgG was obtained from ICN Pharmaceuticals. Anti–mouse CD48 rat monoclonal antibody MRC OX-78 was obtained from Serotech. Anti–*Escherichia coli* GroEL mouse monoclonal antibody 9A1/2 was obtained from Calbiochem. Anti–mouse CagA biotin-labeled mouse monoclonal antibody has been described (6, 12). Anti–mouse CD48 rat monoclonal antibody (Becton Dickinson) or Brucella broth containing 1.5% sheep sera has been described (35). Anti–LAMP-1 rat monoclonal antibody 1D4B was obtained from the Developmental Studies Hybridoma Bank of the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine and the Department of Biology, University of Iowa.

**Bacterial Strains and Media.** All *B. abortus* derivatives were derived from 544 (ATCC23448), smooth virulent *B. abortus* biovar 1 strains. Ba598 (544 *ΔvirB4*), Ba600 (544 GFP*^+^*), and Ba604 (*ΔvirB4* GFP*) have been described (6, 14). *B. abortus* strains were maintained as frozen glycerol stocks and cultured on Brucella broth (Becton Dickinson) or Brucella broth containing 1.5% sheep sera. Kanamycin was used at 40 μg/ml.

**Construction of An In-Frame Deletion Mutant of virB2.** pMAW24 (*ΔvirB2*) was constructed by cloning two PCR fragments into Sall/Sacl-cleaved pSR47s (14). Fragment 1 was a 1,609-bp Sall–BglII fragment spanning a site located 1,609 nucleotides upstream of the 5′ end of *virB2* to 6 nucleotides downstream from the 5′ end and was amplified by PCR using primers 5′-GTCGACATGCAGCATTATCCAAACCTC-3′ (Sall site underlined) and 5′-AGATCTTCTCATGACTTATCCCTAAA-3′ (BglII site underlined; nucleotide positions 1 and 1,614 are available from GenBank/EMBL/DDBJ under accession no. AF226278, respectively; reference 10). Fragment 2 was a 1,600-bp BamHI–SacI fragment spanning the region starting 6 nucleotides upstream of the 3′ end of *virB2* to a position 1,594 nucleotides downstream from the 3′ end and was amplified using primers 5′-GGATCCAGATGCAGCATTATCCAAACCTC-3′ (BamHI site underlined) and 5′-GAGCTCCATCCCGTTCGCTGGCGGAGCAGACATCAT-3′ (Saci site underlined; nucleotide positions 1,921 and 3,526 are available from GenBank/EMBL/DDBJ under accession no. AF226278, respectively; reference 10). pMAW24 (*ΔvirB2*) was introduced into *E. coli* DH5α (*p15T*), and then the plasmid was transferred into *B. abortus* 544 by electroporation (Gene Pulser; Bio–Rad Laboratories). Isolation of in-frame deletion mutant by the positive selection for sucrose resistance has been described (14).

pMAW25 (*virB2*) was constructed by cloning a PCR fragment into Sall/BamHI-cleaved pBBR1MCS-2 (15). The 707-bp EcoRI–BamHI PCR fragment spanning a site located 369 nucleotides upstream of the 5′ end of *virB2* to a position 21 nucleotides downstream from the 3′ end (10) and was amplified using the primers 5′-GTCGACGTATTAGCCGGCGGCGCCGAC-3′ (Sali site underlined) and 5′-GGATCCGGTTGTACGATCATTGCTCCCTC-3′ (BamHI site underlined).

**Cell Culture.** Bone marrow–derived macrophages from female BALB/c, C57BL/6, Nsgk, or Zrcr PrP*^C−^*–deficient mice (16, 17), and PrP*^C−^* transgenic Nsgk PrP*^C−^*–deficient mice (18) were prepared as previously described (6, 14). After culturing in L-cell–conditioned medium, the macrophages were replated for use by lifting cells in PBS on ice for 5 to 10 min, harvesting cells by centrifugation, and resuspending cells in RPMI 1640 containing 10% fetal bovine serum. The macrophages were seeded (2–3 × 10^6^ per well) in 24-well tissue culture plates for all assays.

**Immunofluorescence Microscopy.** Detection of intracellular bacteria, macrophosome formation, and fluorescence–labeled molecules by fluorescence microscopy have been described (6). In brief, *B. abortus* strains were grown to A600 = 3.2 in Brucella broth and used to infect mouse bone marrow–derived macrophages for various lengths of time at a multiplicity of infection of Hsp60 rabbit polyclonal antibody was obtained from MBL International Corporation. Anti–glucose–6–phosphate dehydrogenase (G6PDH) goat polyclonal antibody was obtained from Cortex Biochem. *Brucella*-infected human, cattle, and sheep sera have been described (13). Anti–LAMP-1 rat monoclonal antibody 1D4B was obtained from the Developmental Studies Hybridoma Bank of the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine and the Department of Biology, University of Iowa.
20. Infected cells were fixed in periodate-lysine-parafomaldehyde containing 5% sucrose for 1 h at 37°C. Samples were washed three times in PBS and wells were successively incubated three times for 5 min in blocking buffer (2% goat serum in PBS) at room temperature.

All antibody-probing steps were for 1 h at 37°C. Samples were washed three times in PBS for 5 min and then permeabilized in −20°C methanol for 10 s. After incubating three times for 5 min with blocking buffer, samples were stained with each primary antibody. After washing three times for 5 min in blocking buffer, samples were stained simultaneously with each secondary antibody. Samples were placed in mounting medium and visualized by fluorescence microscopy.

100 macrophages were examined per coverslip to determine the total number of intracellular bacteria, macropinosome formation, and total number of bacteria within macropinosomes (6).

**Determination of Efficiency of Bacterial Uptake and Intracellular Growth by Cultured Macrophages**. To determine uptake of bacteria, mouse bone marrow–derived macrophages were infected with *B. abortus*. After 0, 5, 15, 25, and 35 min incubation at 37°C, macrophages were washed once with medium and incubated with 30 μg/ml gentamicin for 30 min. Macrophages were then washed three times with fresh medium and lysed with distilled water. CFUs were determined by serial dilutions on Brucella plates. Percentage protection was determined by dividing the number of bacteria surviving the assay by the number of bacteria in the infectious inoculum, as determined by viable counts.

To determine intracellular growth of bacteria, the infected macrophages were then washed once with medium and incubated with 30 μg/ml gentamicin. At different time points, cells were washed and lysed with distilled water and the number of bacteria was counted on plates of a suitable dilution (6).

**Ni-NTA Agarose Pull-Down and Immunoprecipitation Assay**. A fusion protein of Hsp60 tagged with six histidine residues at the NH2 terminus was constructed using the QIAexpress system with pQE30 plasmid (QIAGEN). The fusion Hsp60 protein was purified by Ni-NTA chromatography.

For the pull-down assay, Ni-NTA agarose beads–bound Hsp60 (20 μg/ml) were added to 1 ml macrophage lysate (∼109 cells) prepared with lysis buffer (10 mM Tris–HCl, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 1% Triton X-100, 0.1% SDS, 4 μg/ml leupeptin, 1 mM PMSF; reference 19), and the mixture was incubated at 37°C for 20 min. Ni-NTA agarose beads–bound PrP C (20 μg/ml; reference 20) were added to 1 ml purified Hsp60 solution (20 μg/ml), and the mixture was incubated at 37°C for 20 min.

For immunoprecipitation assay, 20 μg/ml Hsp60 added to 1 ml macrophage lysate was incubated at 37°C for 20 min. The sample was then immunoprecipitated with the anti-PrP C antibody and incubated at 4°C overnight. Protein A–Sepharose beads were added to the sample and incubated at room temperature for 1 h. Each protein or antibody (20 μg/ml) was added in reaction solution and incubated for 20 min before pull-down or immunoprecipitation for binding inhibition.

The precipitates were washed with PBS and analyzed by immunoblotting with either anti–Hsp60 or anti-PrP C, and silver staining was performed using 2D-Silver Stain II (Daicichi Pure Chemicals).

**Expression of Hsp60 on Lactococcus Lactis**. pMAW30 (B. abortus Hsp60+) or pMAW31 (E. coli Hsp60+) was constructed by cloning a PCR fragment into KpnI/SacI–SacI-cleaved pSECE1, which is a vector for the secretion of foreign protein to the cell surface of *L. lactis* (21). The 1,640-bp KpnI-SacI or 1,647-bp SacI PCR fragment spanned the hsp60 gene of *B. abortus* (22) or *E. coli* (23) and was amplified using the primers 5′-GGTACCATGGTCAAAAAAGACGTAAG-3′ (KpnI site underlined) and 5′-GGAGCTTATTAGAAGTCCGCGCAT-3′ (SacI site underlined), or 5′-GGAGCTATTGCGACGTTAAGACGTA-AAA-3′ (SacI site underlined) and 5′-GGAGCTTACCATGGCGCCCATGCC-3′ (SacI site underlined). Transformation of *L. lactis* IL1403 was performed according to the method of Holo and Nes (24).

**Hsp60 Localization on Bacteria**. Bacteria were grown to A600 = 3.2 in broth, collected by centrifugation, and fixed in 4% paraformaldehyde. Expression of Hsp60 on the *B. abortus* or *L. lactis* surface was confirmed by immunofluorescence microscopy with anti-Hsp60 monoclonal antibody (25). Immunofluorescence staining of permeabilized bacteria was performed as previously described (25).

**ELISA**. The ability of PrP C to bind to Hsp60 on *L. lactis* was measured as follows. A 50-μl aliquot of ∼109 *L. lactis* was placed into 96-well immuno plates (Nunc) and incubated at room temperature for 2 h. The sample was then removed and the wells were washed twice with PBS-0.05% Tween 20. 50 μl macrophage lysate (200 μg/ml) were added and the plate was incubated at 37°C for 1 h. The amount of bound PrP C was determined by ELISA with anti-PrP C antibody.

**Time Lapse Video Microscopy**. Bone marrow–derived macrophages were plated in Lab-Tek Chambered coverslip (Nunc) and incubated overnight in RPMI 1640 containing 10% FBS at 37°C in 5% CO2. 2 × 104/ml bacteria were added to the chamber and then the chamber was placed on a heated microscope stage set to 37°C for observation using an Olympus IX70 inverted phase microscope with 100× UPlanApo lens fitted with phase contrast optics. The bacteria were allowed to settle passively onto the macrophages and images were captured over a 30-min period. The images were captured every 15 s using a cooled CCD camera (CoolSNAP; Roper Scientific) and processed using Openlab software (Improvision) on a Power Macintosh G4 computer.

**Virulence In Mice**. Virulence was determined by quantitating the survival of the strains in the spleen after 10 d. Groups of five mice were injected intraperitoneally with ∼104 CFUs of brucella in 0.1 ml saline. At 10 d after infection, their spleens were removed and homogenized in saline. Tissue homogenates were serially diluted with PBS and plated on Brucella agar to count the number of CFUs in each spleen.

**Results**

**Tail Formation of PrP C with Swimming Internalization of B. abortus**. Our previous results showed that GPI-anchored proteins were selectively incorporated into macropinosomes containing *B. abortus* (Fig. 1; reference 6). To investigate further the membrane sorting process, the distribution of GPI-anchored proteins during swimming internalization of *B. abortus* was analyzed. Aerolysin from *Aeromonas hydrophila*, which binds to the GPI moiety of GPI-anchored protein, was observed at the same time as the internalization of PrP C for 20 min. To investigate further the membrane sorting process, the distribution of GPI-anchored proteins during swimming internalization of *B. abortus* was analyzed. Aerolysin from *Aeromonas hydrophila*, which binds to the GPI moiety of GPI-anchored protein, was observed at the same time as the internalization of PrP C for 20 min.
point (Fig. 1). Similar results were obtained for other GPI-anchored proteins, such as CD55 (unpublished data). However, when one GPI-anchored protein, PrPC, was tested, colocalization of aggregated PrPC tail and swimming bacteria was observed (Fig. 2 A). Sometimes, several PrPC tails were observed from a single bacterium (Fig. 2 A). PrPC was also incorporated into macropinosomes containing wild-type strain, but not virB4 mutant, after 15 min incubation (Fig. 2 A).

To obtain the ratio of PrPC tail formation, colocalization of PrPC tail and internalized bacteria was quantitated microscopically at various times of incubation. virB4 mutant was rapidly internalized, with most bacteria internalized before further incubation, but the internalization of wild-type strain was delayed (Fig. 2 B). Wild-type strain, but not virB4 mutant, was present in macropinosomes transiently (Fig. 2 C). The kinetics and degree of association of the PrPC mutant with internalized wild-type strain showed maximal association after 5 min incubation (Fig. 2 D). The maximal association of PrPC with phagosomes containing wild-type strain was observed after 15 min incubation (Fig. 2 E). In contrast, colocalization of PrPC with virB4 mutant was much less pronounced (Fig. 2 D and E). These results suggested that bacterial products secreted by the type IV secretion system might aggregate PrPC specifically and form tail-like aggregation of GPI-anchored proteins (Fig. 2 A).

To investigate bacterial factors associated with PrPC tail formation, immunodominant proteins were examined by immunoblotting with human brucellosis sera, which recognized a major protein (∼60 kD) and two minor proteins (∼30 kD; Fig. 3 A). In a previous report (22), immunodominant Hsp60 reacted with sera from mice experimentally infected with B. abortus. Because Hsp60 was secreted into the external medium, culture supernatant of B. abortus was analyzed by immunoblotting. Immunoreactive proteins were not detected in culture supernatant (unpublished data). However, surface-exposed Hsp60 on wild-type strain, but not virB2 and virB4 mutants, was detected by immunofluorescence staining with anti-Hsp60 antibody (Fig. 3 C). Because introduction of complementing plasmid into each mutant restored surface expression of Hsp60, the expression of Hsp60 on the bacterial surface associates with the type IV secretion system (Fig. 3 C).

To demonstrate that, as above, the presence of Hsp60 on the bacterial surface did not result from wholesale relocation of cytoplasmic leakage, a control experiment was performed. Surface exposure of G6PDH was determined by immunofluorescence microscopy. Antibody against G6PDH failed to react with bacterial cell surfaces. As it was not certain that the control antibody was able to react with bacterial cells in the immunofluorescence experiment, the antibody was used to probe bacteria in the presence or absence of permeabilization by hypotonic lysozyme treatment (25). Antibody against G6PDH reacted with permeabilized bacteria, but failed to react with bacterial cell surface (Fig. 3 D). Therefore, the surface exposure of Hsp60 is not caused by cytoplasmic leakage.

Interaction of PrPC with Hsp60 of B. abortus. Because Hsp60 expressed on the bacterial surface by the type IV secretion system was most likely interacting with the target cell, we tested Hsp60 for its ability to bind to PrPC on macrophages by pull-down assay with Hsp60 or PrPC beads. Analysis of the precipitated proteins by immunoblotting with anti-PrPC or Hsp60 antibody showed that a 29-kD PrPC was associated with Hsp60, but not beads alone (Fig. 4, A and B). To confirm this association, Hsp60 was added to macrophage lysate and the proteins in the mixture were then immunoprecipitated with anti-PrPC antibody. The
The precipitated proteins were analyzed by immunoblotting with anti-Hsp60 antibody. The precipitates contained Hsp60 (Fig. 4 B). Because the anti-Hsp60 antibody did not recognize macrophages Hsp60, the antibody showed specific for bacterial Hsp60 (Fig. 4 B). This Hsp60 and PrP<sup>C</sup> association was inhibited by the addition of anti-Hsp60 polyclonal antibody, purified Hsp60, or PrP<sup>C</sup> (Fig. 4, A and B). These results indicated that the interaction between Hsp60 and PrP<sup>C</sup> would be specific. The precipitated proteins were also analyzed by silver staining. The precipitates contained two major bands (60 and 29 kD) and two weak minor bands (74 and 27 kD; Fig. 4 C). These results suggested that Hsp60 bound to PrP<sup>C</sup> mostly, but there is possibility that Hsp60 might interact indirectly with PrP<sup>C</sup> mediated by other cellular components.

To further characterize Hsp60, distribution of Hsp60 in B. abortus–infected macrophages was analyzed by immunofluorescence microscopy. At 5 or 15 min after infection, Hsp60 colocalized with only the bacterial surface and was not detected in macrophage membrane or cytoplasm (Fig. 4 D).

To investigate if Hsp60 exposed on bacterial surface could aggregate PrP<sup>C</sup> on macrophages, macrophages were infected with L. lactis expressing Hsp60 of B. abortus on its surface (Fig. 5 B), and then PrP<sup>C</sup> was detected by immunofluorescence microscopy. After 5 min incubation, PrP<sup>C</sup>
accumulated around internalized Hsp60+ L. lactis but not Hsp60− L. lactis (Fig. 5 A). Quantitative data showed that >70% of L. lactis expressing Hsp60 colocalized with PrP<sup>C</sup> at all time points (Fig. 5 D). PrP<sup>C</sup> tail formation was not observed with either Hsp60+ or Hsp60− L. lactis. L. lactis was seeded on the wells of a microtiter plate, macrophage lysate was added, and then binding activity was measured by ELISA with anti-PrP<sup>C</sup> antibody. The binding of PrP<sup>C</sup> to Hsp60 on the L. lactis surface was detected but not with Hsp60− L. lactis (Fig. 5 C). L. lactis expressing Hsp60 of E. coli also colocalized with PrP<sup>C</sup> at all time points, but the percentage of colocalization was lower than Hsp60 of B. abortus (Fig. 5, C–E). These results suggested that Hsp60 expressed on the bacterial surface promoted accumulation of PrP<sup>C</sup>, but is not sufficient for PrP<sup>C</sup> tail formation.

Effect of PrP<sup>C</sup> Deficiency on B. abortus Infection. To investigate the role of PrP<sup>C</sup> on B. abortus infection, several phenotypes of B. abortus virulence were tested by using macrophages from Ngsk PrP<sup>C</sup>-deficient mice (16). Time lapse videomicroscopy was used to follow the internalization of B. abortus by macrophages from parent or Ngsk PrP<sup>C</sup>-deficient C57BL/6 mice. After contact of macrophages with B. abortus, bacteria showed swimming internalization in macrophages from parent mice (Fig. 6 A). The swimming of the bacteria on the macrophage surface lasted for several minutes with generalized plasma membrane ruffling before eventual enclosure in macropinosomes (Fig. 6 A). Contact of B. abortus with macrophages from Ngsk PrP<sup>C</sup>-deficient mice, in contrast, resulted in much smaller ruffling that was restricted to the area near the bacteria. The ruffles associated with internalization of bacteria resulted in a more rapid uptake than observed for macrophages from parent mice (Fig. 6 B). 5 min after deposition on the macrophages from parent mice, B. abortus showed generalized actin polymerization around the site of bacterial binding, which could be observed by either phalloidin staining or phase contrast microscopy (Fig. 6 C). Macrophages from Ngsk PrP<sup>C</sup>-deficient mice showed primarily small regions of phalloidin staining at sites of bacterial binding (Fig. 6 C).

The differences in rate of phagocytosis and macropinosome formation for parent or Ngsk PrP<sup>C</sup>-deficient mice were quantitated microscopically at various times of incubation. The kinetics of bacterial internalization and macropinosome formation in macrophages from parent C57BL/6 mice were almost identical to those observed for macrophages from BALB/c mice (Figs. 2, B and C, and 7, A–C). Internalization of wild-type B. abortus into macrophages from Ngsk PrP<sup>C</sup>-deficient mice, in contrast, was much quicker and macropinosome formation was hardly detectable (Fig. 7, D–F). The internalized wild-type strain did
not replicate in the macrophages from Ngsk PrP<sup>C</sup>-deficient mice (Fig. 7 G). Macrophages from parent and Ngsk PrP<sup>C</sup>-deficient mice showed no significant difference in the internalization, macropinosome formation, and intracellular replication of virB4 mutant (Fig. 7, A–F and H). In macrophages from Ngsk PrP<sup>C</sup>-deficient mice, wild-type strain failed to block phagosome maturation as shown by colocalization of phagosomes containing the bacteria and the late endocytic marker, LAMP-1, at 35 min after infection (Fig. 8, A and C). In contrast, wild-type strain prevented phagosome-lysosome fusion, and therefore phagosomes containing wild-type strain do not have LAMP-1 in macrophages from parent mice (Fig. 8, A and B).

To determine if this defect in intracellular replication of B. abortus correlates with an inability to establish infection in the host, we experimentally infected parent or PrP<sup>C</sup>-deficient mice with B. abortus. Many bacteria were recovered from the spleen of BALB/c and C57BL/6 mice infected with wild-type strain at 10 d after infection, but few bacteria were recovered from PrP<sup>C</sup>-deficient mice, based on the number of CFUs in each spleen (Fig. 7 I). As previously reported (14), fewer bacteria were recovered from the spleen of three mice strains infected with virB4 mutant (Fig. 7 I). These results suggested that replicative phagosome formation and proliferation in mice of B. abortus required the uptake pathway associated with PrP<sup>C</sup>.

Several of the phenotypes ascribed to Ngsk PrP<sup>C</sup>-deficient mice are most likely caused by up-regulation of prion protein (PrP)-like protein doppel rather than by ablation of PrP<sup>C</sup> (27). To investigate the involvement of doppel expression on B. abortus infection, Zrch PrP<sup>C</sup>-deficient mice (17), with no up-regulation of doppel, were used for infection assay. The results showed that phenotypes of Zrch PrP<sup>C</sup>-deficient mice were almost the same as Ngsk PrP<sup>C</sup>-deficient mice on B. abortus infection (Fig. 7 G). In addition, PrP<sup>C</sup> transgenic Ngsk PrP<sup>C</sup>-deficient mice were successfully rescued from the inhibition of bacterial intracellular growth (Fig. 7 G). Therefore, doppel expression was not involved in B. abortus infection.

**Discussion**

In this study, we have shown that Hsp60 of B. abortus, secreted on the bacterial surface by the type IV secretion
system–associated manner, interacted directly or indirectly with PrP C, and that the interaction contributed to establish B. abortus infection. The cellular function of PrP C is unclear. Our results in this study provide a novel aspect of PrP C function as a receptor for an intracellular pathogen. Hsp60s, a member of the GroEL family of chaperonins in E. coli, is widely distributed and conserved between prokaryotes and mammals (28). Hsp60 proteins have been recognized as immunodominant antigens of many microbial pathogens, including B. abortus (22, 29). Hsp60s are believed to reside in the cytoplasm (30). However, surface-exposed Hsp60 has been reported in Legionella pneumophila and shown to be involved in pathogenicity (31). Presumably, Hsp60 of L. pneumophila binds to unknown receptors on nonprofessional phagocyte HeLa cells, initiating actin polymerization and endocytosis of the bacterium into an early endosome (32). But the role of surface-exposed Hsp60 in professional phagocytes, such as macrophages, is still unclear. As L. pneumophila has a type IV secretion system, surface expression of Hsp60 of L. pneumophila might
be a similar mechanism to that of B. abortus. Effector proteins secreted by the type IV system of B. abortus have not been identified and this study is the first report describing a candidate effector-like protein secreted by the type IV system of B. abortus. Hsp60 are major antigens that elicit strong antibody responses in many bacteria (29). This includes bacteria that lack the type IV secretion system. Therefore, there is a possibility that Hsp60 might release by another secretion system and bind a denatured part of an effector protein of the type IV secretion system that might carry the Hsp60 to the bacterial surface.

It has been reported that PrP\textsuperscript{C} interacts with Hsp60 by using a Saccharomyces cerevisiae two-hybrid screening system (33). The PrP is the causative agent of neurodegenerative diseases such as Creutzfeld-Jakob disease in humans, bovine spongiform encephalopathy, and scrapie in sheep (34). The pathological, infectious form, PrP\textsuperscript{Sc}, is a \( \beta \) sheet aggregate, whereas the normal cellular isoform, PrP\textsuperscript{C}, consists of a largely \( \alpha \) helical, autonomously folded COOH-terminal domain and an NH\(_2\)-terminal segment that is unstructured in solution (35). Conformational conversion of PrP\textsuperscript{C} into PrP\textsuperscript{Sc} has been suggested to involve a chaperone-like factor. GroEL of E. coli can catalyze the aggregation of chemically denatured and of folded, recombinant PrP in a model reaction in vitro (36). Based on a previous report, it was thought that surface-exposed Hsp60 of B. abortus could bind to PrP\textsuperscript{C} and catalyze the aggregation of PrP\textsuperscript{Sc} on macrophages. Consistent with this hypothesis, Hsp60 expressed on L. lactis could catalyze the aggregation of PrP\textsuperscript{C} on macrophages. However, PrP\textsuperscript{C} tail formation was not observed in macrophages infected with Hsp60\textsuperscript{+} L. lactis. Hsp60 is not sufficient for PrP\textsuperscript{C} tail formation. In addition, swimming internalization and macropinosome formation were not observed in macrophages infected with Hsp60\textsuperscript{+} L. lactis. PrP\textsuperscript{C} tail formation was required for bacterial swimming on macrophages and another bacterial factor, secreted by the type IV system, appears to be required for PrP\textsuperscript{C} tail formation.

B. abortus internalizes into macrophages by swimming on the cell surface for several minutes, with membrane sorting occurring during this period (6, 37). PrP\textsuperscript{C} tail formation is involved in the signaling pathway for swimming internalization because the PrP\textsuperscript{C} tail colocalized with Grb2 (unpublished data). Recently, evidence that PrP\textsuperscript{C} interacts
with Grb2 was provided by the two-hybrid screening system (38). Grb2 is an adaptor protein involved in intracellular signaling from extracellular or transmembrane receptors to intracellular signaling molecules (39). The structure of Grb2 consists of a central SH2 domain flanked by two SH3 domains. The SH2 domain is responsible for interaction with tyrosine kinase, whereas the SH3 domains can bind to proline-rich motifs (40). Grb2 interacts through its SH3 domains with Wiskott-Aldrich syndrome protein (WASP), which plays a role in regulation of the actin cytoskeleton (41). WASP is a 64-kD protein expressed exclusively in hematopoietic cells (42). The carboxyl terminal portion of WASP contains regions that show homology to several actin-binding proteins, such as verprolin and cofilin, which may allow binding of WASP to filamentous actin (43). In regard to internalization of B. abortus, surface-exposed Hsp60 of B. abortus promotes aggregation of PrP C, and PrP C tail formation is induced by unidentified factor(s) secreted by the type IV system. The interaction of PrP C tail with Grb2 will initiate cytoskeletal rearrangement and induce generalized membrane ruffling. Bacteria may obtain driving force for swimming internalization from membrane ruffling, like riding the wave of membrane until enclosed in macropinosomes. Consistent with this hypothesis, Grb2, which had interacted with PrP C tail, was excluded in macropinosomes containing B. abortus (unpublished data). Presumably, the signal mediated by Grb2 is not required for replicative phagosome formation after macropinosome formation. Instead, a signal mediated by lipid rafts is needed for replicative phagosome formation (6).

The function of the B. abortus virB locus is essential for intracellular survival, both in cultured cells and in the mouse model (10, 11, 44–46). Our results of virulence for mice confirmed these previous works. The role of mouse macrophages in mediating resistance or susceptibility among mouse strains to some intracellular pathogens has been shown by studies of the Ity/Lsh/Bcg resistance model. Resistance to Salmonella enterica serovar Typhimurium, Leishmania donovani, and mycobacterial species is regulated by polymorphism of the Nramp1 gene that controls macrophage function (47). Bovine Nramp1 is a major candidate for controlling the in vivo–resistant phenotype against B. abortus infection (48). Our previous study indicated that Niemann-Pick type C1 gene (NPC1) regulated the inter-
nalization and intracellular replication of *B. abortus* and also contributed to bacterial proliferation in mice (49). Macrophages from NPC1-deficient mice did not support internalization and intracellular replication of *B. abortus* (49). In this study, inhibition of internalization was not observed in macrophages from PrPC-deficient mice. In NPC1-deficient mice macrophages, lipid raft-associated molecules, such as cholesterol, GM1 ganglioside, and GPI-anchored proteins, accumulated only in intracellular vesicles (49). In contrast, these molecules were present in both plasma membrane and intracellular vesicles of macrophages from PrPC-deficient mice as well as macrophages from parent mice (unpublished data). Therefore, lipid raft-associated molecules on the plasma membrane are essential for the internalization of *B. abortus*, and PrP<sub>C</sub> promotes the bacterial swimming internalization.

Lipid rafts are involved in infection by several intracellular pathogens. For example, macropinosomes containing *L. pneumophila* included lipid raft-associated molecules (50). GPI-anchored proteins were present in *Toxoplasma gondii* and *Plasmodium falciparum* vacuoles (51, 52). The intracellular parasite *L. donovani* can actively inhibit the acquisition of flotillin-1–enriched lipid rafts by phagosomes and the maturation of these organelles (53). Lipid platforms have been implicated in the budding of HIV and influenza virus (54, 55). The compartmentalization of Ebola and Marburg viral proteins within lipid rafts during viral assembly and budding has also been shown (56). In addition, PrP was attached to membranes by a GPI-anchor that associated with lipid rafts, and a recent study showed that conversion of raft-associated PrP<sub>C</sub> to the protease-resistant state required insertion of PrP<sup>Sc</sup> into contiguous membrane (57). Thus, lipid rafts, including PrP<sub>C</sub>, may have an important role as a gateway for the intracellular trafficking of pathogens (58).

Current treatment of acute brucellosis requires combined regimen of antibiotics and is conditioned by the fact that brucellae are facultative intracellular pathogen. Thus, it is important to treat patients with drugs that penetrate macrophages. This fact seems to be responsible for the long duration of the disease and the high incidence of relapses. To this end, the study of the immunogenicities of antigens and their use in combination with new systems is very important for the development of better vaccines or antimicrobial agents. New strategies are also necessary to prevent brucel-
loss while avoiding the disadvantages of the currently used live vaccines for animals. The study of host–pathogen molecular interactions raises the possibility of novel vaccines or antimicrobial agents. The results of our study thus provide a potential new target for prevention of infection by intracellular pathogens.

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