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GTPases of the Rho Subfamily Are Required for *Brucella abortus* Internalization in Nonprofessional Phagocytes

DIRECT ACTIVATION OF Cdc42*

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Members of the genus *Brucella* are intracellular *α-Proteobacteria* responsible for brucellosis, a chronic disease of humans and animals. Little is known about *Brucella* virulence mechanisms, but the abilities of these bacteria to invade and to survive within cells are decisive factors for causing disease. Transmission electron and fluorescence microscopy of infected nonprofessional phagocytic HeLa cells revealed minor membrane changes accompanied by discrete recruitment of F-actin at the site of *Brucella abortus* entry. Cell uptake of *B. abortus* was negatively affected to various degrees by actin, actin-myosin, and microtubule chemical inhibitors. Modulators of MAPKs and protein-tyrosine kinases hampered *Brucella* cell internalization. Inactivation of Rho small GTPases using clostridial toxins TcdB-10463, TcdB-1470, TcsL-1522, and TcdA significantly reduced the uptake of *B. abortus* by HeLa cells. In contrast, cytotoxic necrotizing factor from *Escherichia coli*, known to activate Rho, Rac, and Cdc42 small GTPases, increased the internalization of both virulent and non-virulent *B. abortus*. Expression of dominant-positive Rho, Rac, and Cdc42 forms in HeLa cells promoted the uptake of *B. abortus*, whereas expression of dominant-negative forms of these GTPases in HeLa cells hampered *Brucella* uptake. Cdc42 was activated upon cell contact by virulent *B. abortus*, but not by a noninvasive isogenic strain, as proven by affinity precipitation of active Rho, Rac, and Cdc42. The polyphasic approach used to discern the molecular events leading to *Brucella* internalization provides new alternatives for exploring the complexity of the signals required by intracellular pathogens for cell invasion.

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Brucellosis is a contagious bacterial disease of animals and a true zoonosis. It is caused by facultative intracellular organisms of the genus *Brucella*, composed of six recognized species with affinity for different hosts (1–4). Infection in humans depends upon contact with infected animals or their products, causing a severe syndrome that, if left untreated, may lead to disability and death (4). Despite the fact that the first member of the genus was described more than 100 years ago, the intracellular life cycle and virulence mechanisms of *Brucella* are just being unveiled (5–7). In comparison with other pathogenic bacteria, *Brucella* lacks classical virulence factors such as exotoxins, invasive proteases, toxic lipopolysaccharide, capsules, virulence plasmids, and lysogenic phages. Furthermore, it does not generate resistance forms; does not display antigenic variation; and lacks fimbriae, pili, and flagella (8). In general, *Brucella* virulence resides in its well developed ability to invade, survive, and replicate within vacuolar compartments of professional and nonprofessional phagocytes (6, 9–14). In professional phagocytes as well as in caprine M (lymphoepithelial) cells, *Brucella* is ingested by a zipper-like mechanism (15). Opsonized brucellae are internalized via complement and Fc receptors in macrophages and monocytes, whereas non-opsonized brucellae seem to penetrate via lectin or fibronectin receptors, in addition to other unknown receptors (16, 17). In nonprofessional phagocytes, *Brucella* appears to be internalized by receptor-mediated phagocytosis (18, 19). Although zipper-like phagocytosis has been observed in these cells (7), it seems to be more an exceptional event than a common phenomenon (18, 20).

Penetration into nonprofessional phagocytes occurs within minutes after inoculation, with one or two brucellae/cell (6). Cytoskeletal rearrangements have not been directly observed, but these structures seem to be required, since various cytoskeletal chemical modulators hamper the internalization of *Brucella* in these cells (7, 19). Although the molecular mechanisms underlying these phenomena are not known, at least one signaling system, BvrR-BvrS, coding for a regulator (BvrR) and a sensor protein (BvrS) has been implicated in the invasion of *Brucella abortus* into cells (14). In the same vein, the absence of *O*- and native hapten polysaccharides on the *Brucella* surface considerably hampers bacterial cell invasion (14, 17, 21). These type of mutations are known to modify the topology and bio-

logical properties of the *Brucella* outer membrane, altering the attachment to and penetration into host cells (22–24).

The ability of different bacteria to exploit cell signal transduction pathways and cytoskeletal components to secure their survival is a well recognized event. Paradigms of host subversion by either intracellular or extracellular bacteria such as *Salmonella*, *Shigella*, *Listeria*, *Neisseria*, *Yersinia*, and *Escherichia* have been established in recent years (25–31). By interacting with cytoskeletal regulators such as the small GTP-binding proteins of the Rho subfamily, these bacteria have developed efficient ways to induce cytoskeletal rearrangements. GTPases of the Rho subfamily function as molecular switches that cycle between an active GTP-bound state and an inactive GDP-bound state. Activated proteins of the Rho subfamily interact with effector molecules to produce biological responses involving actin reorganization. Some of these responses involve membrane rearrangements implicated in several functions, one of them being phagocytosis (32).

To characterize the basic molecular events that proceed after *B. abortus* binds to nonprofessional phagocytic HeLa cells, several microscopic and biological strategies were followed. Initially, we employed cytoskeletal chemical modulators in cells previous to infection. Then, we used bacterial toxins capable of modifying small GTPases of the Rho family as well as expression of dominant-positive or dominant-negative GTPase forms in cells during bacterial infection. Finally, we performed direct quantification of activated small GTPases after infection with *B. abortus*. The data obtained indicate that *B. abortus* modulates the host cell cytoskeleton to induce its internalization.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—All strains were routinely grown in tryptic soy or Luria-Bertani medium. *B. abortus* 2308 NaI^r is a wild-type virulent smooth lipopolysaccharide strain that has been described elsewhere (33). *B. abortus* 2.13 is a smooth lipopolysaccharide noninvasive 2308 NaI^r derivative with a Tn5 insertion in *bvrS* (14). *Salmonella typhimurium* SL1344 (34) was obtained from Stéphane Méresse (Centre d'Immunologie de Marseille-Luminy). *Escherichia coli* expressing CNF[1]; plasmids encoding Myc epitope-tagged Cdc42V12 and Cdc42N17 derived from pMT90 (from Philipe Chavrier, Institut Curie-Section Recherche, Paris, France); and plasmids expressing Myc epitope-tagged RhoAV14, RhoAN19, Rac1V12, and Rac1N17 derived from pEXV (35, 36) were provided by Gilles Flatau and Patrice Boquet (INSERM, Nice, France). GST-tagged RBD was expressed from plasmid pGEX-2T-TRBD (provided by Xiang-Dong Ren and Martin Alexander Schwartz, Scripps Research Institute, La Jolla, CA) (37). GST-tagged PBD was expressed from a derivative pGEX-2T plasmid (obtained from Gary M. Bokoch, Scripps Research Institute) (38).

Cell Culture, Microinjection, and Transfection—Cells were grown in Eagle's minimal essential medium supplemented with 5% fetal bovine serum, 2.5% sodium bicarbonate, and 1% glutamine. Penicillin (100 units/ml) and streptomycin (100 μg/ml), which were routinely added, were excluded from cell cultures during *Brucella* infections. For cell microinjection, 5×10^5 HeLa cells were seeded on 13-mm glass slides and incubated for 24 h at 37 °C in 5% CO_2. Cells were microinjected (FemtoJet®, Eppendorf) into the nucleus with the selected plasmids at a concentration of 1 μg/ml in sterile distilled water and infected with *B. abortus* as described below. After a 16-h incubation in the presence of 5 μg/ml gentamycin, cells were processed for immunofluorescence. Successfully injected cells and intracellular bacteria were localized by immunofluorescence using an anti-Myc antibody (clone 9E-10, Santa Cruz Biotechnology), a TRITC-conjugated anti-mouse antibody (Sigma), and a bovine FITC-conjugated anti-*Brucella* antibody (39). Cell transfection was carried out in 24-well tissue culture plates using Lipofectin (Life Technologies, Inc.) according to the manufacturer's instructions. *Brucella* cell infections were performed as described below.

Binding and Invasion Assays—HeLa cells were grown to subconfluency in 24-well tissue culture plates at 37 °C under 5% CO_2. Chemical cytoskeletal modulators (Sigma) listed in Table I were present throughout the experiments and used at concentrations and incubation times according to Rosenshine *et al.* (40). The chemical 2,3-butanedione monoxime was used at a concentration of 7 nM for 30 min (41); PD098059 was used at a concentration of 50 μM for 40 min (42); and wortmannin was used at a concentration of 50 nM for 30 min (43). TcdB-10463, TcdB-1470, TcdA, and TcsL-1522 selective toxin inhibitors of small GTPases were prepared as described (44). *E. coli* CNF was purified according to Falzano *et al.* (45). Unless otherwise stated, the toxin working concentrations and incubation times used were as follows: 50 ng/ml TcdB-10463 for 40 min, 50 ng/ml TcdB-1470 for 40 min, 5 ng/ml TcdA overnight, 1 μg/ml TcsL-1522 overnight, and 3 ng/ml CNF for 2 h. Intoxication of HeLa cells was always carried out prior to *B. abortus* infection. After intoxication, the monolayer was washed once with cold phosphate-buffered saline (0.01 M, pH 7.4) and kept at 4 °C until infection. Infections were carried out using an overnight culture of *B. abortus* diluted in Eagle's minimal essential medium to reach a concentration of 2.5×10^8 cfu/ml. The inoculum was then added to the monolayer at a multiplicity of infection of 500 cfu/ml. For *Salmonella* control experiments, the multiplicity of infection was 50 cfu/ml. Plates were centrifuged at $300 \times g$ at 4 °C, incubated for 30 min at 37 °C under 5% CO_2, and washed three times with phosphate-buffered saline. Extracellular bacteria were killed by adding Eagle's minimal essential medium supplemented with 100 μg/ml gentamycin for 1 h at 37 °C under 5% CO_2. Plates were then washed with phosphate-buffered saline. HeLa cells were lysed by adding 0.1% Triton X-100 for 10 min. The samples were collected, spun, and resuspended in 110 μl of tryptic soy broth. Aliquots were plated on tryptic soy agar and incubated at 37 °C for 3 days for determination of cfu.

Immunofluorescence and Transmission Electron Microscopy—For immunofluorescence analysis, HeLa cells (5×10^5) were seeded on 13-mm glass slides, incubated until subconfluent at 37 °C under 5% CO_2, and inoculated with bacteria as described above. After five washing steps with phosphate-buffered saline, cells were fixed with ice-cold 3% paraformaldehyde (Merck) for 15 min. Samples were washed once and incubated for 10 min with phosphate-buffered saline containing 50 mM NH_4Cl. Intracellular and extracellular bacteria were detected and counted as previously described (11). Briefly, extracellular bacteria were labeled using a FITC-conjugated anti-*Brucella* antibody diluted 1:250 (in 10% horse serum in phosphate-buffered saline), followed by washing steps. Intracellular bacteria were detected by incubating the slides for 30 min with rabbit anti-*B. abortus* antiserum (39) diluted 1:250 in 10% horse serum containing 0.1% saponin (permeabilization step). The cells were then washed three times with 0.2% Tween 20 and incubated for 30 min with a TRITC-conjugated anti-rabbit antibody (Jackson ImmunoResearch Laboratories, Inc.) diluted 1:150 in 10% horse serum containing 0.1% saponin. When needed, FITC-phalloidin (Sigma) was added at this point. Slides were mounted in Mowiol solution and analyzed by phase-contrast or fluorescence microscopy. Counts of intracellular and extracellular bacteria were performed in at least 100 infected cells and are expressed as the mean ± S.D. of bacteria/cell. The percentage of cells with associated bacteria is expressed as the mean ± S.D. of cells with bound bacteria in five different 40× fields. Statistical analysis was performed using Student's *t* test. For transmission electron microscopy, HeLa monolayers infected with an overnight culture of *B. abortus* 2308 NaI^r were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer. Samples were placed in 1% OsO_4 solution for 1 h for post-fixation, dehydrated in a graded concentration of ethanol, and infiltrated with Spurr resin. Thin sections on 300 mesh collodion-coated grids were stained with uranyl acetate and lead Sato's solution (46). Preparations were examined with a Hitachi H-7100 electron microscope operating at 75 kV.

Quantification of GTP-Rho, GTP-Rac, and GTP-Cdc42—For precipitation steps, GST-tagged RBD and PBD were purified from cell lysates of *E. coli* strains harboring plasmids pGEX-2T-TRBD and pGEX-2T-PBD, respectively, according to Ren *et al.* (37) and Benard *et al.* (38). HeLa cells grown in six-well plates were infected for different time intervals with *B. abortus* at a multiplicity of infection of 5000 cfu/cell. After incubation, cells were washed with ice-cold phosphate-buffered saline and lysed with 500 μl of ice-cold precipitation buffer (1% Triton X-100, 0.1% SDS, 0.3% Nonidet P-40, 500 mM NaCl, 10 mM $MgCl_2$, and 50 mM Tris, pH 7.2). Lysates were clarified by centrifugation at 14,000 rpm for 1 min. Twenty μl of lysate were saved as a control of total GTPase content. GTP-loaded Rho GTPases were precipitated with

[1] The abbreviations used are: CNF, cytotoxic necrotizing factor from *E. coli*; GST, glutathione *S*-transferase; RBD, Rhotekin Rho-binding domain; PBD, GTPase-binding domain of p21-activated kinase-1; FITC, fluorescein isothiocyanate; cfu, colony-forming units; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; TRITC, tetramethylrodamine isothiocyanate; TcdB, *Clostridium difficile* toxin B; TcdA, *C. difficile* toxin A; TcsL, *C. sordellii* lethal toxin.

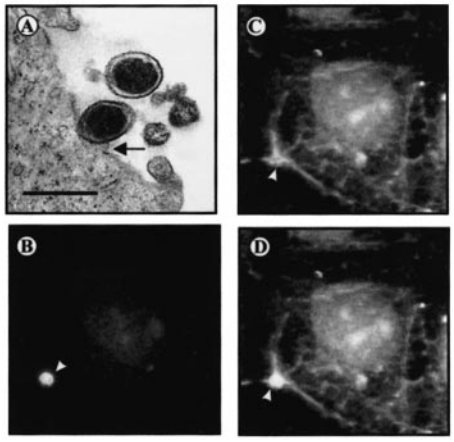

FIG. 1. ***B. abortus* induces minor cytoskeletal rearrangements in HeLa cells.** *A*, transmission electron microscopy of *B. abortus* entry into HeLa cells reveals discrete cellular projections at the site of contact between the cell and bacterium (*arrow*). *Bar* = 0.4 μm. *B–D*, double immunofluorescence analysis of F-actin and extracellular *B. abortus* bound to HeLa cells. In *B*, the *arrow* points to *B. abortus* immunolabeled with rabbit anti-*Brucella* antiserum and TRITC-conjugated anti-rabbit IgG serum after cell infection. In *C*, the *arrow* points to foci of actin polymerization stained with FITC-phalloidin. In *D*, the superimposition of *B* and *C* demonstrates colocalization of *B. abortus* and actin rearrangement.

Sepharose beads coupled to either GST-PBD or GST-RBD protein. Samples were incubated for 30 min at 4 °C with shaking, washed with precipitation buffer, and resuspended in 25 μl of sample buffer for SDS-polyacrylamide gel electrophoresis analysis (47). Samples transferred to a polyvinylidene difluoride membrane (Roche Molecular Biochemicals) were tested either with rabbit antibodies against Rho or Cdc42 (Santa Cruz Biotechnology) or with an anti-Rac monoclonal antibody (Transduction Laboratories). Probing and developing were performed with peroxidase-labeled secondary antibodies and with a chemiluminescence Western blotting kit (Pierce SuperSignal West Dura), respectively. GTP-Cdc42, GTP-Rho, and GTP-Rac levels were calculated using Scion Image for Windows and compared with control total Cdc42, Rho, and Rac.

RESULTS

Host Cell Cytoskeleton Responds to B. abortus Contact—To assess the role of the host cell cytoskeleton in *Brucella* internalization, HeLa cells were infected with bacteria and analyzed by transmission electron and immunofluorescence microscopy. In agreement with previous investigations (11, 48), few cells in a monolayer had associated bacteria (see below). At 30 min of infection, bacteria were mostly located in cell-cell contacts rather than in the cell body (see below). Minor host cell membrane projections were observed upon contact with bacteria (Fig. 1*A*). Under these experimental conditions, zipper-like phagocytosis was not observed, despite that a considerable number of intracellular brucellae were already found within vacuoles, as previously reported (6). When infected cells were stained with FITC-phalloidin, a discrete rearrangement of the actin cytoskeleton was observed at the site of contact between *Brucella* and its host cell (Fig. 1, *B–D*). To further identify eukaryotic components required for *B. abortus* uptake, HeLa cells were treated with different cytoskeletal and signal transduction modulators before infection with *B. abortus*. Inhibition of the eukaryotic microtubule network with colchicine or nocodazole reduced *Brucella* internalization to 40 and 10%, respectively, compared with non-intoxicated cells (Fig. 2). Treatment of cells with drugs affecting the actin cytoskeleton also impaired internalization. Particularly, cytochalasin D almost abrogated *Brucella* uptake. These results are in agreement with the observations made by electron and fluorescence microscopy, indicating participation of the host actin cytoskeleton in *Brucella* uptake. When tyrosine kinase inhibitors such as tyrphostin and genistein were used, the percentages of internalized bacteria were reduced to 10 and 20%, respectively, compared with untreated cells. Pretreatment of HeLa cells with the MAPK kinase inhibitor PD098059 resulted in a 50% decrease in bacterial invasion, whereas pretreatment with the phosphatidylinositol 3-kinase inhibitor wortmannin reduced *Brucella* internalization to 10%. *S. typhimurium* SL1344 was included as a control of our test system. Fig. 2 demonstrates that the effects induced by the various chemicals modulators were similar to those reported elsewhere for *Salmonella* (Table I).

B. abortus Internalization Is Affected by Modulation of GTPase Activity by Bacterial Toxins—Clostridial toxins TcdB-10463, TcdB-1470, TcsL-1522, and TcdA have been described as glucosyltransferases targeting different members of the Rho and Ras subfamilies of small GTPases (49, 50). They efficiently block the interaction of Rho and Ras protein subfamilies with their effectors, leading to functionally inactive GTPases (51). On the other hand, CNF from *E. coli* exerts the opposite effect, *i.e.* activation of Rho GTPases (52, 53). Since these toxins are very specific for different small GTPases involved in cytoskeleton functions such as membrane ruffling, lamellipodia and stress fiber formation (51, 54), they can be used to study the role of Rho proteins in the internalization of different pathogens (55, 56). HeLa cells treated for 40 min with TcdB-10463 and TcdB-1470 or overnight with TcdA and TcsL-1522 exhibited decreased *Brucella* internalization compared with untreated cells (Fig. 3*A*). In contrast, when cells were treated with CNF for 2 h, an ~10-fold increase in internalization was obtained compared with untreated cells (Fig. 3*B*). We concluded from these experiments that some of the toxin targets outlined in Fig. 3 are relevant for *Brucella* uptake. Because Rho proteins have been implicated in the regulation of the actin cytoskeleton, it was important to determine whether the observed inhibitory effect was due to the direct action of the toxins on Rho proteins or to a secondary effect inducing actin depolymerization. HeLa cells were treated with a constant dose of toxin for different time periods and infected with *B. abortus*. A marked reduction in *Brucella* uptake was seen already after 15 min of intoxication with TcdB-10463 and TcdB-1470 compared with untreated cells (Fig. 4*A*). Since a cytopathic effect was not evident until 30–45 min of intoxication, we concluded that the reduced internalization of *Brucella* was not caused by secondary actin depolymerization. With CNF, increased internalization was observed after 1 h treatment, with a peak at 2–3 h. Membrane ruffling was evident after 2 h of treatment (Fig. 4*B*). The percentage of internalization dramatically decreased after 3 h, probably due to secondary effects such as unavailability of free actin monomers.

CNF (but Not TcdB) Cell Intoxication Affects Adhesion of B. abortus—Successful bacterial invasion depends on two consecutive steps: binding and internalization (57). Inhibition or promotion of *B. abortus* uptake in toxin-treated cells compared with non-intoxicated cells may be due to altered binding and/or internalization. To distinguish between these possibilities, double immunofluorescence to resolve intracellular from extracellular bacteria in cells treated with TcdB-10463 and CNF was performed, and counts were compared with infected non-intoxicated cells (Fig. 5). Binding was not affected by intoxication with TcdB-10463 for 15 min since the mean number of bacteria/cell was not significantly different between non-intoxicated and intoxicated cells ($p > 0.05$). However, the proportion of extracellular to intracellular bacteria was higher in treated cells ($p < 0.05$) (Fig. 5*A*, *panel a*). At 40 min of intoxication, 100% of the cells exhibited some degree of typical arborizing cytopathic effect induced by this toxin (Fig. 5*B*, *panel b*, *TcdB-10463*) as

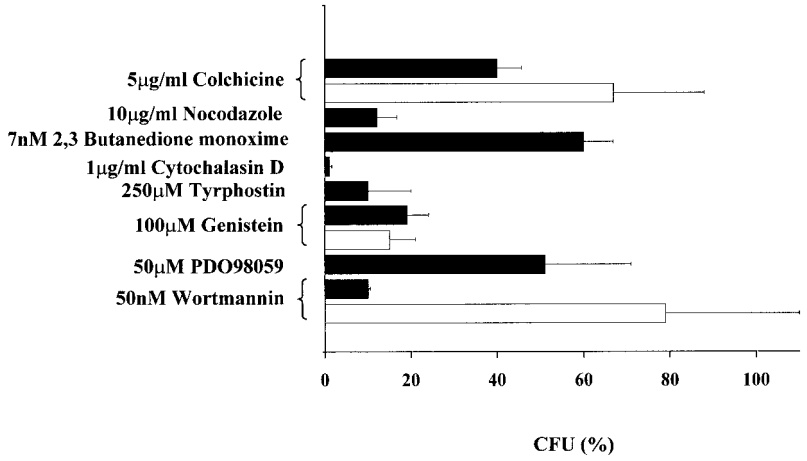

FIG. 2. ***B. abortus* internalization is impaired by chemical cytoskeletal modulators.** HeLa cells were treated with different chemical drugs and then infected with *B. abortus* (*black bars*) or *S. typhimurium* (*white bars*). The effect on bacteria uptake was assessed using the gentamycin survival assay as described under "Experimental Procedures." Mean values of one representative experiment from at least three independent assays were normalized relative to the cfu obtained in infected non-intoxicated cells.

TABLE I
Comparative inhibition pattern of entry for Listeria and Salmonella

Drug	Target	Effect on *Listeria* internalization	Effect on *Salmonella* internalization
Colchicine	Microtubules	Inhibition in macrophages, but not in HT-29 or Caco-2 enterocytes (99, 100).	Not affected in CHO,[a] HEp-2, MDCK, HT-29, Caco-2, and human epithelial cells (100–102).
Nocodazole	Microtubules	Inhibition in macrophages and nonproliferative HT-29 and IPI-2I cells (96, 99).	Not affected in HeLa, MDCK, and human epithelial cells (102–104).
2,3-Butanedione monoxime	Actin-myosin interaction	ND	ND
Cytochalasin D	Actin filaments	1–33% internalization in HeLa cells; inhibition in endothelial, Caco-2, and HT-29 cells; inhibition in HEp-2 cells (96, 100, 105–109).	Inhibition in HeLa, MDCK, CHO, HEp-2, Caco-2, and epithelial cells; increased internalization in HT-29 and Caco-2 cells (100–104, 110).
Tyrphostin	Protein-tyrosine kinases	10–100-Fold inhibition in epithelial intestinal cell lines (111).	Not affected in HeLa cells (112).
Genistein	Protein-tyrosine kinases	10–100-Fold inhibition in intestinal and epithelial cell lines; 47% internalization in endothelial cells; inhibition in macrophages and Caco-2 and HT-29 cells (106, 108, 109, 111, 113, 114).	Not affected in HeLa, Henle 407, and A431 cells; inhibition in Caco-2 and HT-29 enterocytes (40, 114).
PD098059	MAPKs	25% internalization in HeLa cells (108).	Not affected in HeLa cells or macrophages (55, 108).
Wortmannin	Phosphatidylinositol 3-kinase	25% internalization in HeLa cells; 1–2% internalization in Vero cells (43, 108).	Mild inhibition in Vero cells; inhibition of phagocytosis (43, 55).

[a] CHO, Chinese hamster ovary; MDCK, Madin-Darby canine kidney; ND, no data.

described previously (58). Under these conditions, bacteria were found mainly at the edges of the cell body, whereas in control cells, they were found in cell-cell contacts (Fig. 5*B*, *panels a–c*, *Control*, and *TcdB-10463*). Since body retraction is more evident in these intoxicated cells, it was easier to observe the preferential binding of bacteria to the remaining cell-cell contacts. After 40 min of intoxication with TcdB-10463, the mean number of bacteria/cell was not significantly different ($p > 0.05$) from that in control cells (Fig. 5*A*, *panel a*), and the proportion of extracellular bacteria was even higher than in cells intoxicated for 15 min. It has been reported that the percentage of *B. abortus*-infected cells in HeLa cell monolayers is <50% (11, 48). We therefore analyzed whether this percentage is somehow modified in intoxicated HeLa cells. Fig. 5*A* (*panel c*) shows that the percentage of cells associated with bacteria in TcdB-10463-treated monolayers was lower than in non-intoxicated monolayers. In our experiments, the percentages ranged from 10 to 20% in infected non-intoxicated cells and were 6.5 and 3.6% in monolayers treated with TcdB-10463 for 15 and 40 min, respectively, showing that toxin treatment decreases infection. Altogether, these results indicate that binding of *B. abortus* to HeLa cells is not significantly affected by TcdB-10463 intoxication. However, internalization is reduced because less bacteria were taken up per cell, and less cells in the monolayer had associated bacteria. Similar experiments were performed in CNF-treated HeLa cells. Membrane ruffling was recorded after 2 h of intoxication, and bacteria were observed on the cell body (Fig. 5*B*, *panels a–c*, *CNF*), particularly close to ruffles. Electron transmission microscopy of CNF-treated HeLa cells infected with *Brucella* indicated that the bacteria were able to penetrate through membrane ruffles, when present (data not shown). Adhesion of virulent *B. abortus* 2308 to HeLa cells was promoted by CNF treatment compared with untreated cells ($p < 0.05$) (Fig. 5*A*, *panel b*). However, the proportion of intracellular and extracellular bacteria did not differ between control and intoxicated cells ($p > 0.05$). The increased binding was not specific for the virulent strain because the internalization-deficient strain, 2.13 (14), also bound more to CNF-treated cells than to untreated cells ($p < 0.05$). With strain 2.13, however, the ratio of intracellular to extracellular bacteria was increased because more bacteria were found intracellularly (Fig. 5*A*, *panel b*). Therefore, CNF-intoxicated HeLa cells promoted both binding and internalization of non-virulent strain 2.13. With virulent strain 2308, no difference in the ratio of intracellular to extracellular bacteria was observed after 30 min of incubation, despite the fact that binding was promoted. On the other hand, the percentage of cells associated with bacteria was significantly higher ($p < 0.01$) in CNF-treated cells for both the virulent and non-virulent *B. abortus* strains (Fig. 5*A*, *panel d*). In conclusion, CNF

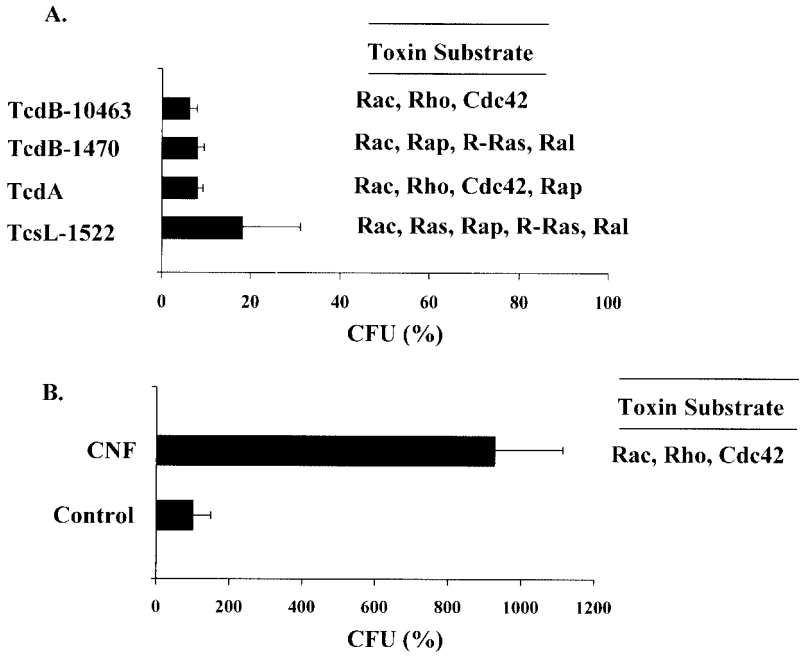

FIG. 3. **Uptake of *B. abortus* by HeLa cells treated with different bacterial toxins.** *A*, gentamycin survival assay of cells treated with different clostridial toxins; *B*, gentamycin survival assay of cells treated with CNF. Mean values of one representative experiment from at least three independent assays were normalized relative to the cfu obtained in infected non-intoxicated cells.

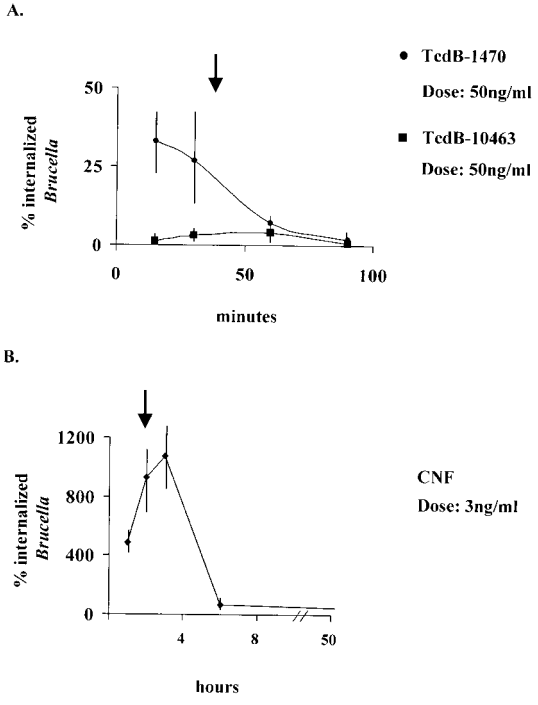

FIG. 4. **Effect on *B. abortus* uptake in TcdB- or CNF-intoxicated HeLa cells occurs before cytopathic effect is evident.** *A*, gentamycin survival assay using TcdB-1470- or TcdB-10463-treated HeLa cells at different time periods; *B*, gentamycin survival assay using CNF-intoxicated HeLa cells at different time periods. The *arrows* indicate the first time that cytopathic effect was evident. Bacteria were incubated with cells after toxin treatment at each time point.

treatment of HeLa cells promotes greater *Brucella* binding per cell and increases the number of cells with associated bacteria, leading to an overall more efficient invasion of the cell monolayer.

B. abortus Internalization Is Affected by the Expression of Dominant-positive or Dominant-negative Rho GTPases—To further investigate the role of small GTPases in *Brucella* uptake, infections of HeLa cells expressing active forms of Rho, Rac, and Cdc42 were performed. HeLa cells were microinjected with plasmids encoding Myc-tagged dominant-positive mutants of Rho, Rac, and Cdc42. *B. abortus* 2308 was incubated for 30 min, followed by the addition of gentamycin to kill extracellular bacteria. After 16 h of gentamycin incubation, when bacterial replication is still not evident in control cells (6), infected monolayers were processed for immunofluorescence. Expression of the corresponding mutant Rho protein was verified using immunofluorescently labeled anti-Myc antibodies as shown in Fig. 6*A*. The number of intracellular bacteria/cell increased in cells expressing positive mutant Rac and Rho (but not Cdc42) compared with control cells (Fig. 6*B*, *panel a*). However, the percentage of cells with internalized bacteria increased in all cases (Fig. 6*B*, *panel b*). As expected, the expression of dominant-negative mutant Rho proteins (RhoAN19, Rac1N17, and Cdc42N17) in transfected HeLa cells inhibited the internalization of this bacterium to different extents (Fig. 7), supporting a role for these small GTPases in *Brucella* uptake.

Cdc42 Is Directly Activated by Virulent (but Not by Non-virulent) B. abortus—The experiments described above indicated that active Rho, Rac, and Cdc42 promote *Brucella* uptake by HeLa cells. However, it was important to establish whether binding of *B. abortus* to HeLa cells leads to direct activation of any of the Rho proteins. Lysates from cells infected with either the virulent 2308 or noninvasive 2.13 strain were incubated with beads bearing the Rho effector RBD or the Rac and Cdc42 effector PBD, according to the affinity capture systems developed by Ren *et al.* (37) and Benard *et al.* (38), respectively. After protein elution, samples were analyzed by Western blotting using anti-RhoA, anti-Rac, or anti-Cdc42 antibodies. Fig. 8*A* shows that no difference in Rho or Rac activation was detected up to 60 min of infection with the virulent 2308 strain. On the contrary, increased levels of GTP-Cdc42 (up to 4-fold) were detected at 30 min of infection (Fig. 8*B*). Cdc42 activation was specific for the virulent strain since the internalization-deficient 2.13 strain did not activate Cdc42 up to 60 min after infection. We therefore concluded that early direct Cdc42 activation is biologically important for successful *B. abortus* internalization.

DISCUSSION

Different attempts have been made to characterize the host-parasite interactions that prevail during *Brucella* entry into eukaryotic cells. Pathological and microscopic studies have

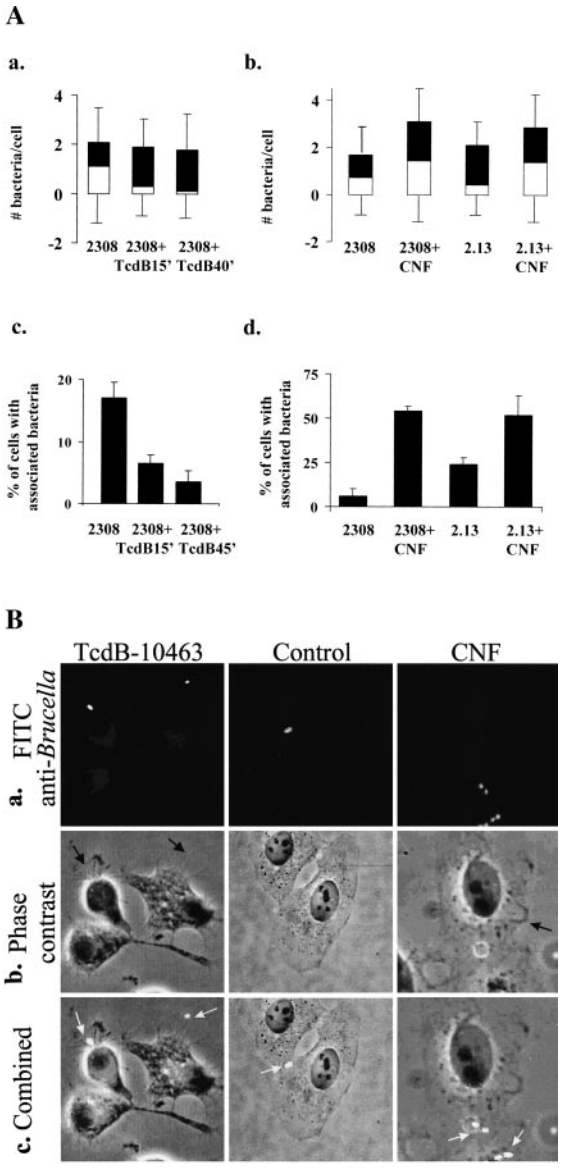

FIG. 5. **Adhesion of virulent *B. abortus* to HeLa cells is not affected by TcdB-10463, but is promoted in CNF-intoxicated HeLa cells.** *A*, HeLa cells were intoxicated with TcdB-10463 for 15 (*15′*) or 40 (*40′*) min or with CNF for 2 h, infected with *B. abortus* for 30 min, and then extracellular (*black bars*) and intracellular (*white bars*) bacteria were counted by double immunofluorescence analysis. *Panel a*, total number and proportion of intracellular/extracellular bacteria/cell in TcdB-10463-intoxicated and non-intoxicated HeLa monolayers; *panel b*, total number and proportion of intracellular/extracellular bacteria/cell in CNF-intoxicated and non-intoxicated cells for both the virulent *B. abortus* 2308 and nonpathogenic 2.13 strains; *panel c*, number of cells with associated bacteria in TcdB-10463-intoxicated and non-intoxicated HeLa cells; *panel d*, number of cells with associated bacteria in CNF-treated and untreated HeLa cells. Counts of intracellular and extracellular bacteria were performed in at least 100 infected cells and are expressed as the mean of bacteria/cell obtained from one representative experiment of three independent assays. The percentage of cells with associated bacteria is expressed as the mean of cells with bound bacteria in five different 40× fields. The results presented are from one experiment of at least two independent assays. *B*, HeLa cells were intoxicated with TcdB-10463 for 40 min or with CNF for 2 h, infected with *B. abortus* for 30 min, and then processed for immunofluorescence. *Panels a*, extracellular bacteria immunolabeled with an FITC-conjugated anti-*Brucella* antibody; *panels b*, bacterial toxin cytopathic effect showing spikes in TcdB-10463-treated cells (*TcdB-10463*, *arrows*) and ruffles in CNF-intoxicated cells (*CNF*, *arrow*) as revealed by phase-contrast microscopy; *panels c*, superimposed images showing *B. abortus* attached to spikes of TcdB-10463-treated cells (*TcdB-10463*, *arrows*) or several bacteria bound to CNF-treated cells displaying membrane ruffles (*CNF*, *arrows*). Bacteria lying between the boundaries of cell-cell contacts (*Control*, *arrow*) are shown.

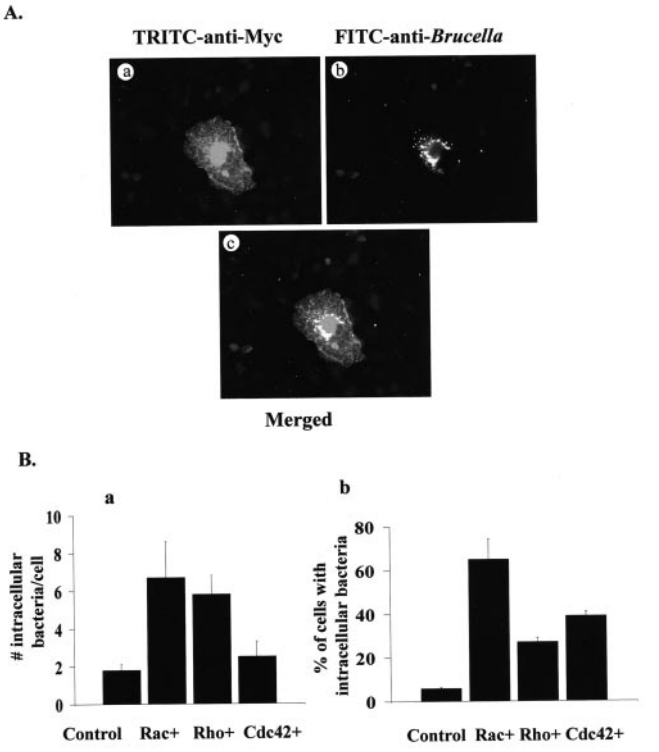

FIG. 6. ***B. abortus* internalization is enhanced in HeLa cells expressing dominant-positive mutants of small GTPases.** *A*, HeLa cells were microinjected with a plasmid encoding the Myc-RhoAV14 fusion protein and infected with *B. abortus* for 30 min. Cells were then fixed, permeabilized, and processed for double immunofluorescence. *Panel a*, microinjected cells had an altered morphology and were evident after immunolabeling using a monoclonal anti-Myc antibody and a TRITC-conjugated anti-mouse antibody. *Panel b*, shown are immunolabeled bacteria using a FITC-conjugated anti-*Brucella* antibody. *Panel c*, merged *panels a* and *b* demonstrate colocalization of transformed cells with *Brucella*. Similar results were obtained when HeLa cells were microinjected with plasmids encoding the Myc-Rac1V12 and Myc-Cdc42V12 fusion proteins (data not shown). *B*, shown are the number of bacteria/cell and proportion of cells with intracellular bacteria in cells expressing dominant-positive mutants of small GTPases. *Panel a*, mean number of intracellular bacteria/cell found in at least 150 microinjected cells. *Panel b*, percentage of cells expressing different dominant-positive mutants with intracellular bacteria. The results presented are from one experiment of at least two independent assays.

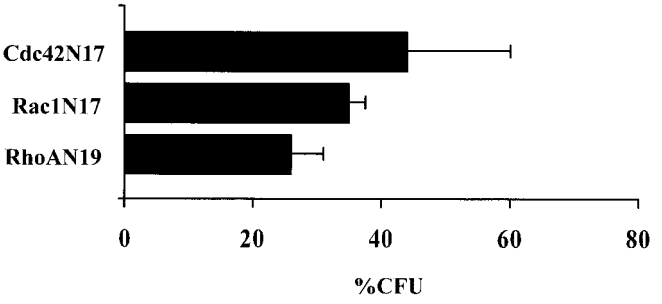

FIG. 7. **Expression of dominant-negative mutants of small GTPases in HeLa cells decreases *B. abortus* internalization.** HeLa cells were transfected with plasmids encoding the Myc-RhoAN19, Myc-Rac1N17, and Myc-Cdc42N17 fusion proteins and infected with the virulent *B. abortus* 2308 strain. The gentamycin survival assay was then performed. Mean values are normalized relative to the cfu obtained in non-transfected cells. The results presented are from one experiment of at least two independent assays.

been reported (15, 18, 59, 60), but the molecular mechanisms involved in the process have not been properly addressed. Evident membrane rearrangements have been described upon *Brucella* infection of caprine M (lymphoepithelial) cells and

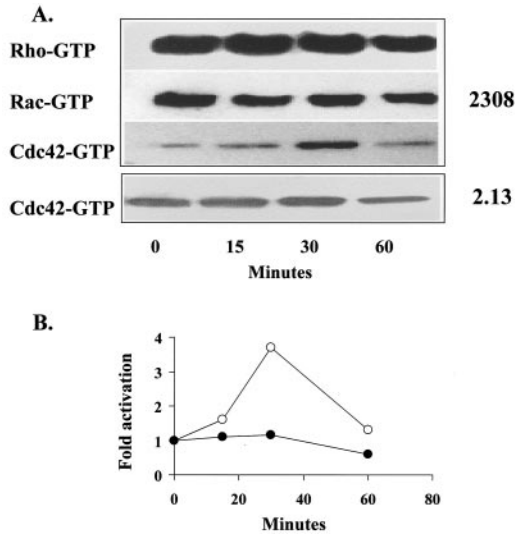

FIG. 8. **Virulent *B. abortus* strain 2308 activates Cdc42 in HeLa cells.** *A*, analysis of activated Rho, Rac, and Cdc42 using affinity precipitation at different times of infection of HeLa cells with virulent *B. abortus* strain 2308 or the isogenic noninvasive mutant strain 2.13. Samples were separated by SDS-polyacrylamide gel electrophoresis, blotted, and immunodetected with anti-Rho, anti-Rac, or anti-Cdc42 antibodies. In the zero time point sample, tryptic soy broth was added to the cells. Samples from lysates were run in parallel on SDS-polyacrylamide gel and immunoblotted using specific anti-small GTPase antibodies to determine the total amount of each GTPase. Increased levels of GTP-Cdc42 were detected after 30 min of infection with the virulent 2308 strain. No differences in the quantities of GTP-Rho and GTP-Rac were detected upon *Brucella* infection. *B*, quantification of Cdc42-GTP levels upon cell interaction with the virulent 2308 (○) and non-virulent 2.13 (●) *B. abortus* strains compared with the negative control. One representative experiment from three different assays is presented.

macrophages (15, 20). Our electron microscopy studies confirmed the results obtained earlier (7, 18), where only slight membrane rearrangements were found at the site of virulent smooth lipopolysaccharide *Brucella* entry into nonprofessional phagocytes. Moreover, phalloidin staining demonstrated a modest recruitment of the F-actin cytoskeleton at the site of attachment. The participation of the actin cytoskeleton was further indicated by reduced internalization of *Brucella* after treatment of HeLa cells with the actin-depolymerizing agent cytochalasin D or with the myosin inhibitor 2,3-butanedione monoxime. Although less dramatic than cytochalasin D, microtubule-depolymerizing agents also hampered the invasion of *Brucella* into cells. Other investigators have arrived at similar conclusions using cytoskeletal inhibitors (7, 19). However, it must be pointed out that this inhibition could be the result of the indirect microtubule inhibitor effect on the MAPK pathway (61–64), which is required for *Brucella* internalization, as shown here.

Uptake of different bacteria depends on the actin cytoskeleton (65–75). Although examples of bacteria requiring only the microtubule network for successful internalization are rare (76), there are many bacteria that recruit both microtubules and microfilaments (77–84). In this respect, *B. abortus* appears to belong to the latter group. Given the growing evidence for potential interactions between the microtubule and actin networks, it is feasible that pathogens exploiting one network would also be dependent on the other (85–87). Involvement of host kinases, particularly protein-tyrosine kinases, in *Brucella* internalization was suggested by the reduced internalization of bacteria by HeLa cells intoxicated with protein-tyrosine kinase-specific drugs such as tyrphostin and genistein. Furthermore, according to the results obtained with PD098059-intoxicated cells, the ERK pathway also appears to be required for *Brucella* uptake to some extent, indicating that *Brucella* is able to trigger a response in its host cell upon contact. Phosphatidylinositols are also involved in this process, as suggested by the decreased entry of *B. abortus* into cells pretreated with wortmannin. Phosphatidylinositol 3-kinase has been shown to be both an upstream and downstream effector of small GTPases (88–90), affecting actin polymerization that eventually could lead to a GTPase-dependent *Brucella* internalization event. A converging molecule for all the pathways studied herein is Ras, a small GTPase activated upon ligand binding to its membrane receptor (particularly tyrosine kinase receptors), coupling intracellular signal transduction pathways to changes in the external environment. There is enough evidence to select the Raf-MEK-MAPK pathway as a key effector in Ras signaling (54). On the other hand, phosphatidylinositol 3-kinase can bind to GTP-Ras (91), and there is evidence that Ras and Rho GTPases interact and are activated in series (32). It would then be relevant to test whether Ras is needed for *Brucella* invasion. According to the results obtained with the chemical drugs, this transduction pathway could be similar to the one exploited by *Listeria*, which appears to be different from the one used by *Salmonella* (Table I). This idea is in agreement with the slight actin recruitment induced by *Listeria* and *Brucella*, but not by *Salmonella*, which induces a major recruitment (26, 67, 69).

Gentamycin survival assays using bacterial toxin-treated cells demonstrated that Rho, Rac, and Cdc42 are needed for efficient *Brucella* internalization. This is also supported by the reduction of bacteria entry into cells expressing dominant-negative mutants of Rho, Rac, and Cdc42 GTPases. Cdc42 (but not Rac or Rho) was directly activated upon *B. abortus* contact with host cells, an event exclusively observed with the virulent strain. Since some clostridial toxins affecting *Brucella* invasion do not use Cdc42 as substrate, it is feasible to conclude the participation of other GTPases from these experiments. In this sense, it is possible that *Brucella* does not directly activate Rho and Rac as well as other Ras proteins, but takes advantage of activated GTPase pools kept in cells under normal conditions. The increase in *B. abortus* uptake observed after cell treatment with CNF and the significant increase observed in cells microinjected with positive forms of Rac and Rho support this asseveration. Nevertheless, other GTPases such as Ral and Rap, implicated in endocytosis (92–94), could be involved in the internalization process as well.

It is important to point out that both TcdB-10463 and TcdB-1470 use the same cell receptor and display very similar enzymatic parameters during cell intoxication. However, these two toxins differ in their substrate preference (49): although TcdB-10463 modifies Rho, Rac, and Cdc42, TcdB-1470 uses Rac as the only member of the Rho subfamily. *B. abortus* internalization is affected earlier by TcdB-10463 intoxication than by TcdB-1470 intoxication as shown by the time curves obtained with these two toxins. Whereas this observation supports the participation of the three GTPases from the Rho subfamily during *B. abortus* internalization, the almost 100% inhibition of *B. abortus* by TcdB-1470 at later times reflects the importance of Rac. Indeed, Rac has recently been described as a potential link between the microtubule and actin networks since microtubule growth induces Rac activation and therefore lamellipodium formation (87).

The results obtained from the intoxication time curves prove that not only the toxin kinetics, but also the physiology of the small GTPases should be taken into account when using this kind of tool. Once bound to their target, the toxins block Rho GTPases in either a GTP- or GDP-bound state. In each of these states, these GTPases have different downstream effects that

are time-dependent. It is important to evaluate the intoxication output at early times, when the direct effects of the toxins on their Rho targets are more likely to be observed than the downstream effects of the small GTPase-intoxicated state. This is clearly exemplified by CNF-treated cells for periods longer than 3 h (Fig. 4*B*).

Binding of *B. abortus* to HeLa cells was not affected by TcdB-10463 treatment for 15 or 40 min. However, according to the gentamycin survival assay, TcdB-10463 treatment for 40 min affected *B. abortus* uptake. Double immunofluorescence experiments indicated that bacteria were binding to cells, but fewer numbers were internalized, and fewer numbers of cells had associated bacteria, explaining this phenomenon. CNF cell intoxication affected *Brucella* invasion in different ways: (i) increased binding of bacteria per cell, with an absolute increase in intracellular bacteria; (ii) increased internalization in the case of the *B. abortus* 2.13 mutant strain, with more intracellular bacteria than in control experiments; and (iii) increased percentage of cells permissive to *B. abortus* internalization. The 10-fold increase in internalization observed in the gentamycin survival assay should be the sum of these events, where probably the augmented number of infected cells has a major contribution. This permissibility event is affected by toxin treatment, suggesting that GTPases of the Rho subfamily might have either a direct or indirect role, perhaps by controlling the formation of cell-cell contacts where *B. abortus* binds or by regulating the expression of a protein particularly found in these regions and required for bacteria to bind. More studies are needed to clarify why bacteria are found mainly in cell-cell contacts and why some cells in the same monolayer are more permissive to *B. abortus* invasion than others, an event also described for *Campylobacter jejuni* and *Listeria* (95, 96).

B. abortus cell uptake may induce a particular signal transduction pathway where small GTPases are activated in series. Indeed, Ras has been reported as a Cdc42 activator, and Cdc42 itself has been described as a Rac activator, whereas Rac activates or inhibits Rho to varying degrees (88, 97, 98). Although the events leading to *Brucella* internalization may follow a similar GTPase activation pathway, this may be a simple view of a more intricate set of signals occurring during the invasion of intracellular pathogens into cells.

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GTPases of the Rho Subfamily Are Required for *Brucella abortus* Internalization in Nonprofessional Phagocytes: DIRECT ACTIVATION OF Cdc42

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