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Tyrosine Phosphorylation Is Required for Ehrlichial Internalization and Replication in P388D$_1$ Cells

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Replication of *Ehrlichia risticii* was inhibited in P388D$_1$ cells when a protein tyrosine kinase inhibitor (genistein or herbimycin A) was added after internalization of the organism at 3 h postinfection. Upon addition of genistein at day 1, 2, 3, or 4 postinfection, further proliferation of *E. risticii* was prevented. The inhibition was reversible, since regrowth of *E. risticii* occurred upon the removal of genistein. Genistein prevented spreading of *E. risticii* from P388D$_1$ cells to THP-1 cells. Genistein did not prevent binding of [^{35}S]methionine-labeled *E. risticii* to P388D$_1$ cells but did prevent internalization of [^{35}S]methionine-labeled *E. risticii*. $^{14}CO_2$ production from L-[^{14}C]glutamine in Percoll density gradient-purified *E. risticii* was not inhibited by genistein or herbimycin A, which suggests that these reagents did not directly inhibit ehrlichial energy metabolism. Double indirect immunofluorescence labeling with antiphosphotyrosine antibody and anti-*E. risticii* antibody revealed colocalization of tyrosine phosphoproteins with ehrlichial inclusions. There was, however, no colocalization of phosphotyrosine with phagosomes containing 0.5-μm-diameter fluorescent beads. Western immunoblot analysis revealed that 52- and 54-kDa proteins were tyrosine phosphorylated only in infected cells and that phosphorylation of these two proteins was reduced when infected cells were treated with genistein for 6 h. These results suggest that protein tyrosine phosphorylation is specific and essential for ehrlichial internalization, replication, and spreading in macrophages but not for binding.

Ehrlichia risticii is a small, obligatorily intracellular, gram-negative bacterium that belongs to the family *Rickettsiaceae* (10). *E. risticii* multiplies in membrane-bound inclusions of monocytes and macrophages by inhibiting lysosomal fusion and causes a disease called Potomac horse fever in equids (10). Internalization of *E. risticii* into mouse macrophages or P388D$_1$ cells was completely inhibited by monodansylcadaverine but was resistant to or only partially inhibited by cytochalasins (8, 11), which suggests that receptor-mediated endocytosis, rather than phagocytosis, is the major mode of entry. Ehrlichial internalization, proliferation, and spreading to new host cells are inhibited by Ca^{2+} channel blockers, Ca^{2+} ionophore, calmodulin antagonists, and inhibitors of intracellular Ca^{2+} mobilization (12), indicating that Ca^{2+} and calmodulin are involved in these processes. Inhibition of lysosomal fusion is reversible, since after treatment of infected cells with oxytetracycline for 30 min, lysosomal fusion with ehrlichial inclusion takes place (22). Inhibition of lysosomal fusion is selective for ehrlichial inclusion, since lysosomal fusion with phagosomes containing latex particles coexisting in the same cytoplasm takes place (22). The uptake signal, the receptor for internalization, and the process by which *E. risticii* maintains this cytoplasmic compartment are still unknown. Protein receptor tyrosine phosphorylation functions as an uptake signal in receptor-mediated endocytosis of various ligands (17). Although protein tyrosine phosphorylation is not required for phagocytosis of extracellular bacteria, such as *Staphylococcus aureus* (23), protein tyrosine phosphorylation is required for actin filament-facilitated phagocytosis of at least two facultatively intracellular bacteria. Protein tyrosine kinase inhibitors are reported to inhibit *Yersinia enterocolitica* invasin (an integrin-binding protein)-promoted bacterial uptake by nonphagocytic epithelial (HeLa) cells, although the molecular size and the intracellular location of the tyrosine-phosphorylated protein are unknown (14). Induction of tyrosine phosphorylation of a host 90-kDa protein is involved in the invasion of HeLa cells by enteropathogenic *Escherichia coli* (13). In contrast, the invasion of epithelial cells by *Salmonella typhimurium* is not inhibited by protein tyrosine kinase inhibitors (14, 15). For obligatorily intracellular bacteria, a requirement of protein tyrosine phosphorylation for infection is unknown, although the presence of phosphotyrosine in chlamydial inclusions has been demonstrated (4). In this study, we evaluated whether protein tyrosine kinase inhibitors have any influence on binding, internalization, proliferation, and intercellular spreading of *E. risticii* in macrophages. Localization of tyrosine phosphoproteins was examined in *E. risticii*-infected macrophages by double immunofluorescence labeling, and the molecular size of the tyrosine phosphoprotein was determined by Western immunoblot labeling of infected cells.

MATERIALS AND METHODS

E. risticii. *E. risticii* Illinois (American Type Culture Collection [ATCC], Rockville, Md.) was cultured in the murine macrophage cell line P388D$_1$ (ATCC) in RPMI 1640 medium (GIBCO, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum (FBS; GIBCO) and 1% 200 mM L-glutamine (L-Gln; GIBCO) without antibiotics. When more than 90% of the P388D$_1$ cells were infected, as determined by Diff-Quik staining (Baxter Scientific Products, Obetz, Ohio), the infected cells were suspended in RPMI 1640 medium at a density of 10^6/ml, sonicated at a power setting of 1.5 at 20 kHz for 5 s by a W-380 ultrasonic processor (Heat System, Farmingdale, N.Y.), and centrifuged at 500 × *g* for 5 min. The supernatant, which contained host cell-free *E. risticii*, was used to infect P388D$_1$ cells.

Infection of P388D$_1$ cells. Confluent monolayers of P388D$_1$ cells pretreated with 10 μg of mitomycin C per ml to prevent mitosis of P388D$_1$ cells for 20 min at 37°C in a 25-cm^2 flask were overlaid with host cell-free *E. risticii*. Cultures were incubated for 3 h and then plated in 96-well round-bottom tissue culture plates (Corning, Inc., Corning, N.Y.) at 4 × 10^3 cells per well. The experimental conditions were set to ascertain that more than 90% of the cells were viable throughout the experimental period. Genistein and herbimycin A were obtained

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from Sigma Chemical Co. (St. Louis, Mo.). The reagents were added 3 h or 1, 2, 3, or 4 days after *E. risticii* was added. To evaluate reversibility, genistein added at day 1 postinfection was removed at day 2, 3, or 4 postinfection and infectivity was determined at day 5 postinfection. To evaluate intercellular spreading, infected $P388D_1$ cells and THP-1 human monocytes (ATCC), which grow in suspension, were preincubated with these reagents for 3 h at 37°C separately and then mixed as described elsewhere (11).

[^{35}S]methionine-labeled *E. risticii* binding and uptake study. Approximately 10^7 *E. risticii*-infected $P388D_1$ cells per ml in 2 ml of methionine- and cysteine-deficient RPMI 1640 medium (ICN Biomedicals, Costa Mesa, Calif.) supplemented with 10% FBS and 2 mM L-Gln were incubated with 2 μM emetine (Sigma) to inhibit eukaryotic protein synthesis at 37°C for 1 h. A metabolic labeling reagent (Tran^{35}S-Label; 11.7 mCi/ml [1,100 Ci/mmol]; 100 μl; ICN Biomedicals) was added, and the mixture was incubated further at 37°C for 24 h. The radiolabeled *E. risticii* cells were released by sonication and washed by centrifugation at 10,000 × *g* for 10 min. To study the attachment of *E. risticii*, radiolabeled *E. risticii* cells (80,000 cpm/20 μl) were added to 6×10^5 $P388D_1$ cells that had been preincubated at 37°C for 3 h with various reagents in 0.4 ml of RPMI 1640 medium containing 10% FBS and 2 mM L-Gln in microcentrifuge tubes and incubated at 4°C for 3 h. The uptake of *E. risticii* was evaluated following removal of bound *E. risticii* cells by incubation with pronase E (Sigma) at 1 mg/ml in phosphate-buffered saline (PBS; 0.14 M NaCl, 8 mM $Na_2HPO_4 \cdot H_2O$, 1.5 mM KH_2PO_4, 2.7 mM KCl, pH 7.4) at 37°C for 10 min after incubation of *E. risticii* with macrophages at 37°C for 4 h. Macrophages were washed three times by centrifugation at 375 × *g* for 5 min per wash, the cells were dissolved in 0.6 N NaOH–0.5% sodium dodecyl sulfate (SDS), and the radioactivity was measured in a scintillation counter.

Effects of genistein and herbimycin A on host cell-free ehrlichiae. *E. risticii*-infected $P388D_1$ cells were disrupted by mild sonication as described above and centrifuged at 600 × *g* for 5 min. The supernatant was centrifuged at 11,670 × *g* for 10 min, and the pellet was suspended in SPK buffer (0.2 M sucrose, 0.05 M potassium phosphate, pH 7.4). The suspension was mixed with Percoll (Sigma) and centrifuged at 61,900 × *g* for 30 min as described by Weiss et al. (21). The lower layer, which contained purified *E. risticii*, was harvested, mixed with SPK buffer, and centrifuged at 11,670 × *g* for 10 min to remove the Percoll. The *E. risticii* pellet was resuspended in SPK buffer, and 0.25 ml of the *E. risticii* suspension was incubated with 50 μl of 1-μCi/ml (231.6-mCi/mmol) L-[^{14}C]glutamine (New England Nuclear Corp., Boston, Mass.) with or without genistein. The tubes were sealed with a rubber stopper top (Kontes Scientific Glassware/Instruments, Vineland, N.J.) from which a plastic center well (Kontes) was suspended. The tubes were incubated in a 37°C water bath with shaking for 2 h, and the reaction was stopped by injecting 250 μl of 25% trichloroacetic acid into the reaction mixture in each tube. Methylbenzethonium hydroxide (1.0 M; Aldrich Chemical Co., Inc., Milwaukee, Wis.) was injected into the center well, and the tubes were further incubated at 37°C in the water bath for 5 min. The methylbenzethonium hydroxide in the well was transferred to a scintillation vial, and Safety-solve High Flash Point Cocktail (Research Products International Corp., Mt. Prospect, Ill.) was added to measure the $^{14}CO_2$ generated in the tube.

Since Percoll interferes with conventional protein assays, the protein in the solution containing purified *E. risticii* in the Percoll gradient was determined by a Coomassie brilliant blue dye-binding assay performed by the method of Vincent and Nadeau (18). To remove Percoll, 100 μl of the *E. risticii* suspension and 100 μl of 0.025% (wt/vol) Triton X-100 (Sigma) in 0.25 N NaOH were centrifuged at 12,000 × *g* for 15 min. The supernatant (50 μl) was mixed with 1 ml of Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, Richmond, Calif.), which was diluted to 1:5 with distilled water. To make a protein standard, bovine serum albumin was prepared in the same solution. The A_{595} was measured in a DU-70 spectrophotometer (Beckman).

Evaluation of infectivity. To estimate infectivity, $P388D_1$ cells in 96-well plates were pipetted into the cytocentrifuge funnel and centrifuged onto a glass slide. Following Diff-Quik staining, the degree of infection was assessed at a magnification of ×1,000. The number of *E. risticii* organisms per cell and the number of infected cells were scored in 100 cells as previously described (11, 12).

Double immunofluorescence labeling. Uninfected and *E. risticii*-infected $P388D_1$ cells (above 70% infectivity and 90% viability by trypan blue dye exclusion assay) were placed into microcentrifuge tubes at 10^5 cells per tube. The cells were fixed in 200 μl of 1% paraformaldehyde in PBS at room temperature for 30 min. The fixed cells were incubated with 5 μg of mouse monoclonal anti-phosphorylated tyrosine immunoglobulin G (IgG) (4G10; Upstate Biotech, Inc., Lake Placid, N.Y.) or of isotype-matched mouse IgG2b (Organon Teknika Corp., West Chester, Pa.) in PBS supplemented with 0.1% gelatin (Bio-Rad) and 0.3% saponin (Accurate Chemical & Scientific Corp., Westbury, N.Y.) per ml at 37°C for 1 h. After three rinses with PBS supplemented with gelatin and saponin, the cells were incubated with Lissamine rhodamine (LRSC)-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Lab., Inc., West Grove, Pa.) at a 1:50 dilution for 1 h at 37°C. The cells were washed as before and incubated with horse (pony 19) polyclonal anti-*E. risticii* serum at a 1:50 dilution for 1 h at 37°C. After the rinse, the cells were stained with a 1:50 dilution of fluorescein isothiocynate (FITC)-conjugated goat anti-horse IgG (Jackson ImmunoResearch) at 37°C for 1 h. The cells were rinsed and resuspended in 100 μl of PBS. The suspension (20 μl) was cytocentrifuged on a glass slide. The cells were mounted in Mowiol 4-88 (Calbiochem, San Diego, Calif.) in accordance with the manufacturer's protocol. Staining was observed under an epifluorescence microscope with an FITC or rhodamine color filter at a magnification of ×1,000. To reduce the background, all secondary antibodies (fluorescent-dye-conjugated antibodies) and pony 19 serum diluted five times in PBS were preabsorbed with sonicated, uninfected $P388D_1$ at room temperature for 2 h at a ratio of 10^6 cells/ml of diluted antiserum. As a control for ordinary phagocytosis, Fluoresbrite plain YG latex beads (diameter, 0.477 ± 0.005 μm; Polysciences Inc., Warrington, Pa.) were used. Uninfected $P388D_1$ cells were transferred into a microcentrifuge tube (10^5 cells per tube) and incubated at 37°C for 1 h with the beads diluted 1:200 in fresh culture medium. The cells were washed with PBS three times by centrifugation at 300 × *g* for 5 min each time. The pellet was fixed with 1% paraformaldehyde for 30 min and subsequently incubated for 1 h with mouse anti-phosphorylated tyrosine IgG diluted 1:50 and for 1 h with LRSC-conjugated goat anti-mouse IgG at 37°C. The coverslips were mounted and viewed under a fluorescence microscope.

Western immunoblotting of tyrosine phosphoproteins. To determine the molecular masses of tyrosine phosphoproteins, SDS-polyacrylamide gel electrophoresis and immunoblotting analysis were used. Uninfected (3×10^5) and *E. risticii*-infected (3×10^5) $P388D_1$ cells were pretreated with 100 μM genistein or 10 μg of oxytetracycline per ml for 6 h at 37°C. After incubation, the cell pellets were suspended in 1× PBS containing 1 mM Na_3VO_4 and 100 mM NaF at room temperature for 10 min. The cells were then sonicated at setting 2 for 10 s and mixed with an equal volume of 2× sample buffer (125 mM Tris, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 10 μg of pyronin Y per ml, pH 6.9) at 1 mg of protein/ml. Samples were boiled at 100°C for 5 min and loaded onto an SDS–10% polyacrylamide minislab gel at 20 μg of protein per lane in a 20-μl volume. A prestained, known-molecular-weight protein mixture (GIBCO-BRL) was loaded onto a separate lane. Electrophoresis was done under constant current (15 mA/gel) for about 2 h in a two-mini gel device (Integrated Separation Systems, Hyde Park, Mass.). Commassie blue staining was used to observe the protein profile of the sample. The protein on the minigel was transblotted onto a BA85 nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, N.H.) at a constant 130 mA for 40 min in a semidry electroblotter (Integrated Separation Systems). The membrane was blocked in blocking solution (5% nonfat dry milk in PBS containing 0.1% Tween 20) at 4°C overnight. The membrane was incubated with mouse monoclonal anti-phosphotyrosine IgG (Upstate Biotech) or with isotype-matched mouse IgG2b (Organon Teknika) as a control at 1 μg/ml in PBS with 0.1% Tween 20 for 2 h at room temperature. After incubation, the membrane was washed three times with PBS supplemented with 0.1% Tween 20. The membrane was subsequently incubated with a 1:1,000 dilution of alkaline phosphatase-conjugated goat antibody to mouse IgG and IgM (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) for 1 h at room temperature. The membrane was washed in PBS containing 0.1% Tween 20 and then incubated with a mixture of alkaline phosphatase substrates (1.65 mg of 5-bromo-4-chloro-3-indolylphosphate and 3.3 mg of nitroblue tetrazolium [Promega, Madison, Wis.]) in 10 ml of color-developing buffer (100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5) at room temperature. The reaction was stopped by rinsing with water. The Western blot result was quantified by using a gel video system (Gel Print 2000i; BioPhotonics Corp., Ann Arbor, Mich.) and image analysis software (ImageQuaNT; Molecular Dynamics, Sunnyvalle, Calif.).

Statistical analyses. Statistical analyses were carried out by using Tukey's studentized range test (9) by means of Minitab statistical software (Minitab Inc., State College, Pa.). $P < 0.05$ was considered significant.

RESULTS

Effects of genistein and herbimycin A on growth of intracellular *E. risticii*. To examine whether replication of intracellular *E. risticii* is inhibited by protein tyrosine kinase inhibitors, these agents were added at 3 h postexposure to *E. risticii*, when most of the ehrlichiae had been internalized (11, 12). Genistein is an isoflavon and inhibits the binding of ATP to the enzyme (1, 2). Herbimycin A is a structurally unrelated, potent, selective protein tyrosine kinase inhibitor. It has direct effects on receptor-associated *src* tyrosine kinases but no effect on protein kinase C or protein kinase A (16). Both agents inhibited ehrlichial replication in $P388D_1$ cells in a dose-dependent manner (Table 1). At a concentration of 100 μM genistein or 100 nM herbimycin A, *E. risticii* infection was almost completely blocked (Table 1 and Fig. 1). This concentration is comparable to those reported for other systems (1, 6). The inhibition is specific to protein tyrosine kinase inhibitors. We previously reported that inhibitors of other kinases, such as H7 (a protein kinase C inhibitor), staurosporine (a serine-threonine kinase inhibitor), or prolonged incubation in phorbol myristate acetate to down-

TABLE 1. Inhibitory effects of genistein and herbimycin A on ehrlichial replication in $P388D_1$ cells[a]

Reagent and concn	% Infected cells	No. of organisms/ 100 cells
None (control)	53 ± 5*	1,228 ± 199†
Genistein		
100 μM	1 ± 1‡	3 ± 6‡
50 μM	9 ± 2‡	102 ± 18§
25 μM	37 ± 65*	510 ± 72§
Herbimycin A		
100 nM	3 ± 1‡	22 ± 13‡
50 nM	22 ± 3§	353 ± 128§
25 nM	46 ± 7*†	648 ± 100§

[a] Reagents were added at 3 h post-*E. risticii* infection and coincubated at 37°C. Infectivity was determined at 72 h posttreatment. Results are the means ± SD of triplicate assays. Identical symbols indicate no significant difference by Tukey's comparisons.

TABLE 2. Effect of genistein on binding and internalization of [^{35}S]methionine-labeled *E. risticii* in $P388D_1$ cells[a]

Reagent	*E. risticii* cpm/6 × 10^5 $P388D_1$ cells[b]	
	4°C for 3 h	37°C for 4 h
None (control)	9,613 ± 1,655*	10,729 ± 1,135*
None plus pronase E	2,396 ± 445†	10,550 ± 1,135*
Genistein (100 μM)[c]	12,696 ± 2,617*	ND[d]
Genistein plus pronase E	ND	2,204 ± 101†

[a] Binding of *E. risticii* was defined as association of organisms with $P388D_1$ cells after 3 h of incubation at 4°C. Internalization was defined as organisms remaining after pronase E treatment at the end of 4 h of incubation at 37°C.
[b] Results are the means ± SD of triplicate assays. Identical superscripts indicate no significant difference by Tukey's comparisons.
[c] Genistein was added 3 h prior to incubation with *E. risticii*.
[d] ND, not determined.

regulate protein kinase C activity, do not inhibit ehrlichial infection at all (12).

Binding and internalization. *E. risticii* specifically binds at 4°C to the host macrophage, and bound ehrlichiae can be removed by brief pronase treatment (8, 11, 12). When *E. risticii* cells are incubated with host macrophages at 37°C, for 3 to 4 h, most of the ehrlichial organisms are internalized and therefore become resistant to pronase treatment (8, 11, 12). It is very difficult to visually count bound or internalized ehrlichial organisms, since very few ehrlichial organisms per macrophage bind and internalize (8). Therefore, as previously described (11, 12), metabolically [^{35}S]methionine-labeled ehrlichiae were used to examine whether tyrosine kinase inhibits ehrlichial binding or internalization. As shown in Table 2, 100 μM genistein did not inhibit binding but did inhibit internalization of approximately 80% of the [^{35}S]methionine-labeled *E. risticii* organisms into $P388D_1$ cells.

Inhibitory effect of genistein added at different days postinfection and reversibility of inhibition. When 100 μM genistein was added at 1, 2, 3, or 4 day postinfection, further growth of cytoplasmic *E. risticii* was prevented regardless of the number of days postinfection (Fig. 1). Withdrawal of genistein allowed regrowth of surviving *E. risticii*, indicating that the inhibitory effect is reversible (Fig. 2).

Effects of genistein on spreading of *E. risticii* from $P388D_1$ cells to THP-1 cells. Ehrlichial organisms apparently spread by endocytosis of organisms exocytosed without cell lysis or liberated by lysis of heavily infected cells (11). To evaluate the involvement of the protein tyrosine kinase in intercellular spreading of ehrlichiae, *E. risticii*-infected $P388D_1$ cells (44% ± 5% infected; 975 ± 122 *E. risticii* cells per 100 $P388D_1$ cells) and uninfected THP-1 cells were left untreated or individually treated with 100 μM genistein for 3 h and then cocultured for 3 days in the absence or presence of reagents, respectively. The

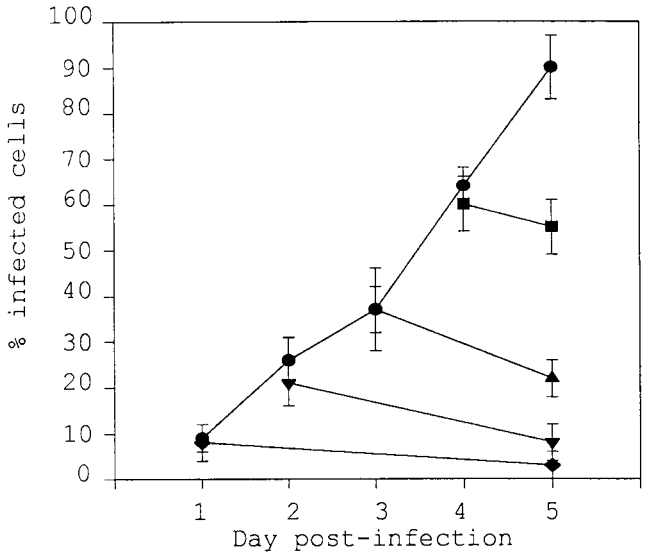

FIG. 1. Inhibitory effect of genistein added at different days postinfection on replication of *E. risticii* in $P388D_1$ cells. Symbols: ●, control (no genistein treatment); ◆, genistein added at 1 day postinfection; ▼, genistein added at 2 days postinfection; ▲, genistein added at 3 days postinfection; ■, genistein added at 4 days postinfection. Infectivity was determined on the day prior to the addition of genistein and at 5 days postinfection. The results are means ± SD of triplicate assays.

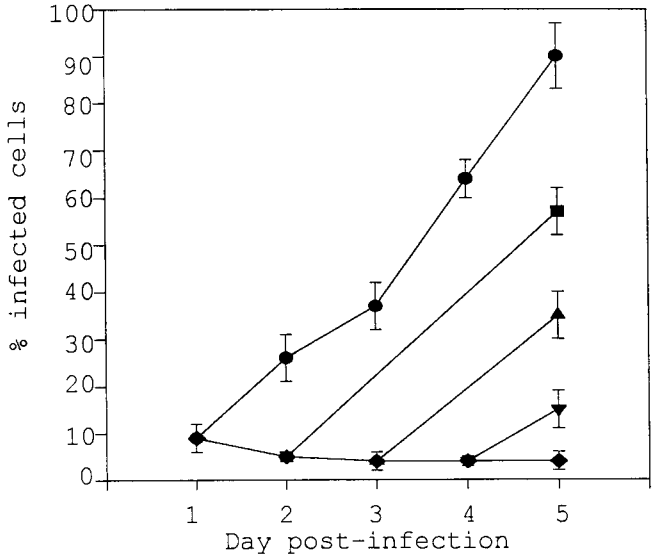

FIG. 2. Inhibitory effect of genistein added to *E. risticii*-infected $P388D_1$ cells at day 1 postinfection and removed after day 2, 3, or 4 postinfection. Symbols: ●, control (no genistein treatment); ■, genistein removed at day 2 postinfection; ▲, genistein removed at day 3 postinfection; ▼, genistein removed at day 4 postinfection; ◆, genistein not removed. Infectivity was determined on the day prior to the removal of genistein and at 5 days postinfection. The results are means ± SD of triplicate assays.

TABLE 3. Effects of protein tyrosine kinase inhibitors on Gln metabolism of Percoll gradient-purified *E. risticii*[a]

Treatment	Amt (μmol) of $^{14}CO_2$ generated/mg of ehrlichial protein/2 h[b]
Sucrose potassium phosphate (no *E. risticii* control)	1.92 ± 0.50*
Sucrose potassium phosphate (control)	6.99 ± 0.83†
Sucrose potassium phosphate + 100 μM genistein	6.56 ± 0.55†
Sucrose potassium phosphate + 200 nM herbimycin A	8.03 ± 0.49†
Sucrose potassium phosphate + 100 nM herbimycin A	7.59 ± 0.35†

[a] Percoll gradient-purified *E. risticii* (26 μg of protein/250 μl) in sucrose potassium phosphate buffer (pH 7.4) was incubated with or without reagents in the presence of 30 mM L-[^{14}C]Gln (52 nCi/50 μl/tube) at 37°C for 2 h.

[b] The $^{14}CO_2$ generated was absorbed in methylbenzethonium hydroxide, and the counts per minute were measured by liquid scintillation counting. Data are the means ± SD of triplicate assays. Identical superscripts indicate no significant difference by Tukey's comparisons.

results (means ± the standard deviations [SD]) of triplicate assays of the control cultures were 31% ± 6% infected THP-1 cells and 384 ± 32 ehrlichiae per 100 THP-1 cells. The corresponding values were both 0 for genistein-treated cultures. Thus, genistein completely prevented intercellular ehrlichial spreading from P388D$_1$ tp THP-1 cells.

Effect of genistein and herbimycin A on host cell-free *E. risticii*. Since ehrlichial organisms are obligatorily intracellular bacteria, it is difficult to determine whether the tyrosine kinase inhibitor directly inhibits *E. risticii* or indirectly inhibits it through inhibition of the host cell tyrosine kinases. Whether *E. risticii* contains tyrosine kinases sensitive to genistein and herbimycin A is unknown. To examine whether the tyrosine kinase directly inhibits ehrlichiae, we preincubated host cell-free *E. risticii* with genistein or herbimycin A at 37°C for 30 min and then determined the infectivity of pretreated ehrlichiae for P388D$_1$ cells in the absence of these reagents after 82 h of incubation. The difference in infectivity between these pretreated *E. risticii* cells and the sham-treated control was not significant (percentages of cells infected: control, 57% ± 5%; 100 μM genistein, 53% ± 4%; 100 nM herbimycin A, 51% ± 4%).

Direct effect of genistein and herbimycin A on energy metabolism of Percoll density gradient-purified ehrlichiae. Host cell-free ehrlichial organisms can generate CO_2 and ATP by metabolizing L-Gln when the assay is done within 3 h after purification (11, 12, 20, 21). The direct effects of genistein and herbimycin A on this ehrlichial metabolic activity were examined. The results showed that ehrlichial energy metabolism was not inhibited by 100 μM genistein or 100 or 200 μM herbimycin A (Table 3).

Colocalization of phosphotyrosine with *E. risticii* inclusion. Double immunofluorescence labeling of *E. risticii*-infected P388D$_1$ cells with antiphosphotyrosine IgG and anti-*E. risticii* serum revealed the colocalization of both markers in approximately 70% of *E. risticii* inclusions (Fig. 3A). There was no significant phosphorylated tyrosine labeling of latex bead (similar in size to ehrlicial organisms)-containing phagosomes in

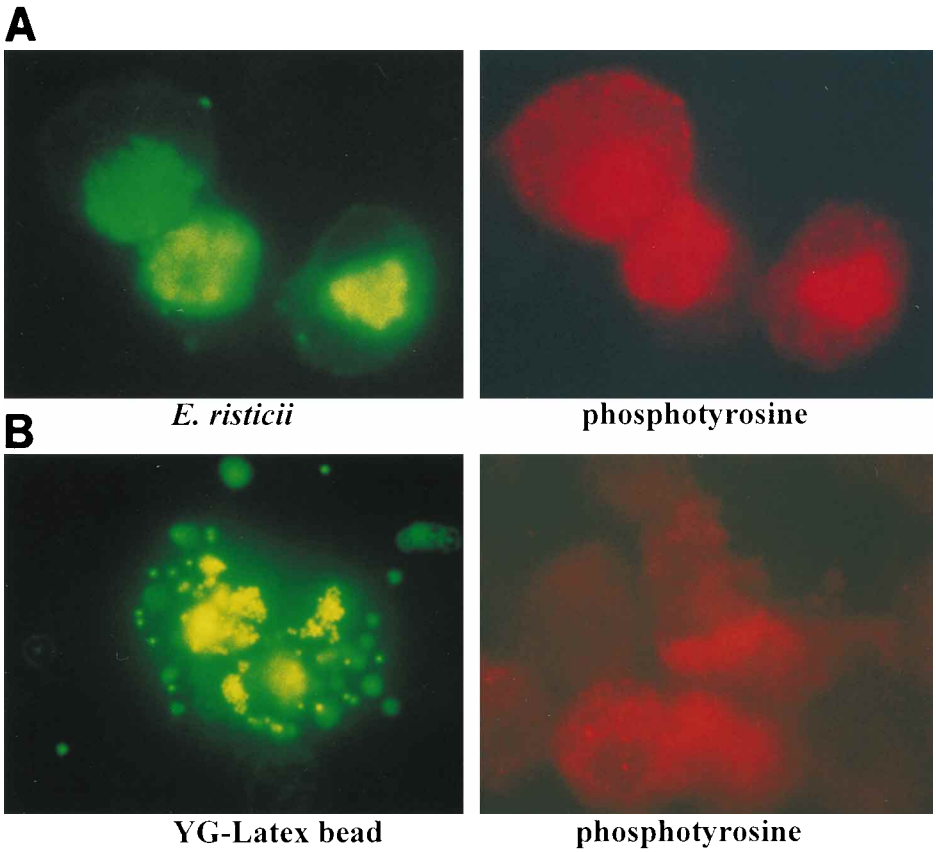

FIG. 3. (A) Colocalization of *E. risticii* inclusions in P388D$_1$ cells stained with anti-*E. risticii* serum and FITC-conjugated anti-horse IgG and phosphotyrosine-labeled with antiphosphotyrosine antibody and LRSC-conjugated anti-mouse IgG. Magnification, ×1,300. (B) There was no colocalization of phosphotyrosine with phagosomes containing latex beads (green fluorescence). Magnification, ×1,300.

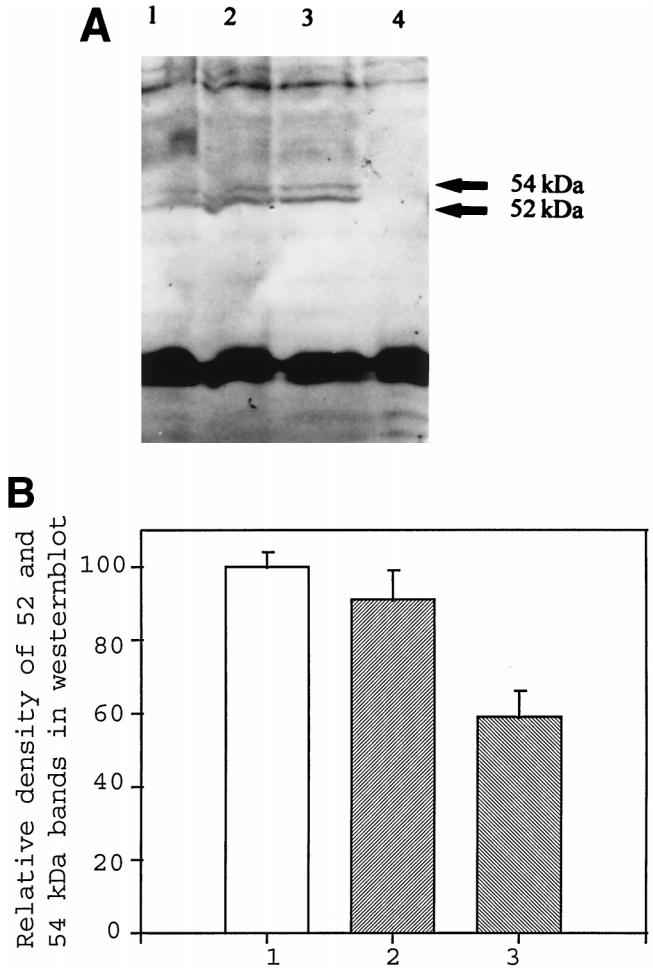

FIG. 4. (A) Western immunoblot analysis with antiphosphotyrosine antibody. Lanes: 1 to 3, *E. risticii*-infected P388D$_1$ cells; 4, uninfected P388D$_1$ cells; 1, treatment with 100 μM genistein for 6 h; 2, treatment with 10 μg of oxytetracycline per ml for 6 h; 3, no treatment. The 52- and 54-kDa proteins were tyrosine phosphorylated only in *E. risticii*-infected cells. (B) Densitometry of 52- and 54-kDa proteins in *E. risticii*-infected P388D$_1$ cells reacted with antiphosphotyrosine antibody by Western immunoblotting. Lanes: 1, control, not treated (set at 100%); 2, treatment with 10-μg/ml oxytetracycline; 3, treatment with 100 μM genistein. The density of uninfected P388D$_1$ cells was set as the background. The results are means ± SD of triplicate assays.

P388D$_1$ cells (Fig. 3B). Isotype-matched mouse IgG2b did not show any reaction to the inclusion membrane (data not shown). The result suggests that ehrlichial inclusion-associated proteins are tyrosine phosphorylated.

Western blot analysis. Western immunoblot labeling revealed that unlike uninfected P388D$_1$ cells, *E. risticii*-infected cells contain two proteins of approximately 52 and 54 kDa that were labeled with antiphosphotyrosine antibody (Fig. 4A). The uninfected cells had constitutively tyrosine-phosphorylated proteins, but these two phosphoproteins were not found. Tyrosine phosphorylation of these two proteins, but not of constitutively tyrosine-phosphorylated proteins, was reduced about 30% in infected cells treated with 100 μM genistein but not in those treated with 10 μg of oxytetracycline per ml for 6 h at 37°C (Fig. 4B). Isotype-matched control mouse IgG2b labeled neither infected- nor uninfected-cell proteins (data not shown). These results suggest that the 52- and 54-kDa proteins are host proteins that must be tyrosine phosphorylated for ehrlichial infection to take place.

DISCUSSION

This study showed that protein tyrosine phosphorylation is essential for ehrlichial internalization, proliferation, and spreading. Genistein and herbimycin A do not inhibit the metabolism of *E. risticii* directly but may inhibit other ehrlichial processes not required for Gln metabolism, or inhibition of the host tyrosine kinase may lead to inhibition of ehrlichial infection. A double immunofluorescence study showed that ehrlichial inclusion-associated proteins are tyrosine phosphorylated. A Western immunoblot study demonstrated that 52- and 54-kDa proteins are tyrosine phosphorylated in *E. risticii*-infected P388D$_1$ cells. Judging from their sensitivity to genistein and resistance to oxytetracycline, these tyrosine phosphoproteins are probably host proteins or ehrlichial proteins that persist after 6 h of oxytetracycline treatment.

The requirement of protein tyrosine phosphorylation for internalization was demonstrated for two facultatively intracellular bacteria: *Y. enterocolitica* (11) and an enteropathogenic strain of *E. coli* (10). For obligately intracellular bacteria, although the *Chlamydia trachomatis* inclusion membrane was shown to be tyrosine phosphorylated (4), the requirement of tyrosine phosphorylation for chlamydial infection was not described. Thus, *E. risticii* is the first obligately intracellular bacterium shown to require tyrosine phosphorylation for infection. Protein tyrosine phosphorylation of growth factor receptors or the Fc receptor is transient. In Fc receptor-mediated phagocytosis of antibody-coated erythrocytes by mouse peritoneal macrophages, immunofluorescence labeling revealed that tyrosine phosphorproteins colocalize with F actin beneath phagocytic cups (6). However, in a short time (<7 min), both phosphotyrosine and F actin staining disappear (6). In contrast, for ehrlichial inclusions, tyrosine phosphoproteins were seen several days after infection, which suggests that ehrlichial inclusions may maintain the early endosome characteristic.

The appearance of immunofluorescence staining, the molecular masses of tyrosine-phosphorylated proteins, and the sensitivity of tyrosine phosphorylation to tyrosine kinase inhibitors were different in *C. trachomatis* and *E. risticii*. During the first 8 h of infection, phosphotyrosine and chlamydial elementary bodies colocalize in HeLa cells (4). At 16 h after infection, elementary bodies have reorganized to the replicating reticulate bodies, and phosphotyrosine is seen as a punctate pattern along the periphery of the inclusion (4). In contrast, staining of *E. risticii* inclusions with antiphosphotyrosine antibody was diffuse and almost completely overlapped the anti-*E. risticii* serum staining. It is not clear why approximately 30% of the *E. risticii* inclusions were not stained with the antiphosphotyrosine antibody. After internalization, approximately one-half of the *E. risticii* organisms go on to proliferate but the remaining organisms die out (8). These ehrlichial inclusions that are not stained with antiphosphotyrosine antibody may be destined to fuse with lysosomes. In another words, tyrosine phosphoprotein may be required for individual inclusions to prevent lysosomal fusion. The γ subunit of Fc γ receptors, P72^{syk}, and paxillin were tyrosine phosphorylated during Fc receptor-mediated phagocytosis in macrophages (7). By Western immunoblot analysis, substrates of the protein tyrosine kinase were 52- and 54-kDa proteins that appear to be host proteins, since oxytetracycline treatment had no influence on these proteins. In agreement with this observation, tyrosine phosphoproteins identical in molecular size were not detected in purified *E. risticii* by Western immunoblot analysis (unpublished data).

During *C. trachomatis* infection of HeLa cells, 64-, 66-, 68-, 94-, and 140-kDa bands are tyrosine phosphorylated 15 min after infection and are continuously phosphorylated throughout 1 day (4). Whether tyrosine phosphorylation of one or all of these protein is required for establishment of infection or is a coincidental phenomenon needs to be clarified. For example, *Salmonella typhimurium* (15) and bacterial lipopolysaccharide (19) induce tyrosine phosphorylation of 42- and 44-kDa mitogen-activated protein kinase, which is not required for bacterial invasion.

C. trachomatis-induced protein tyrosine phosphorylation is not inhibited by genistein (4). On the other hand, reduction of tyrosine phosphorylation of 52- and 54-kDa proteins in *E. risticii*-infected cells after treatment with genistein suggests that tyrosine phosphorylation of these proteins is reversible and is actively maintained by a protein tyrosine kinase. Bacterial protein tyrosine kinases are rare, although tyrosine kinase activity has been demonstrated in two different bacteria (3, 5). Lack of any direct influence of genistein or herbimycin A on ehrlichiae suggests that the protein tyrosine kinase involved in ehrlichial infection is of host origin. Further study is required to characterize the tyrosine-phosphorylated proteins and the protein tyrosine kinases involved in ehrlichial infection.

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